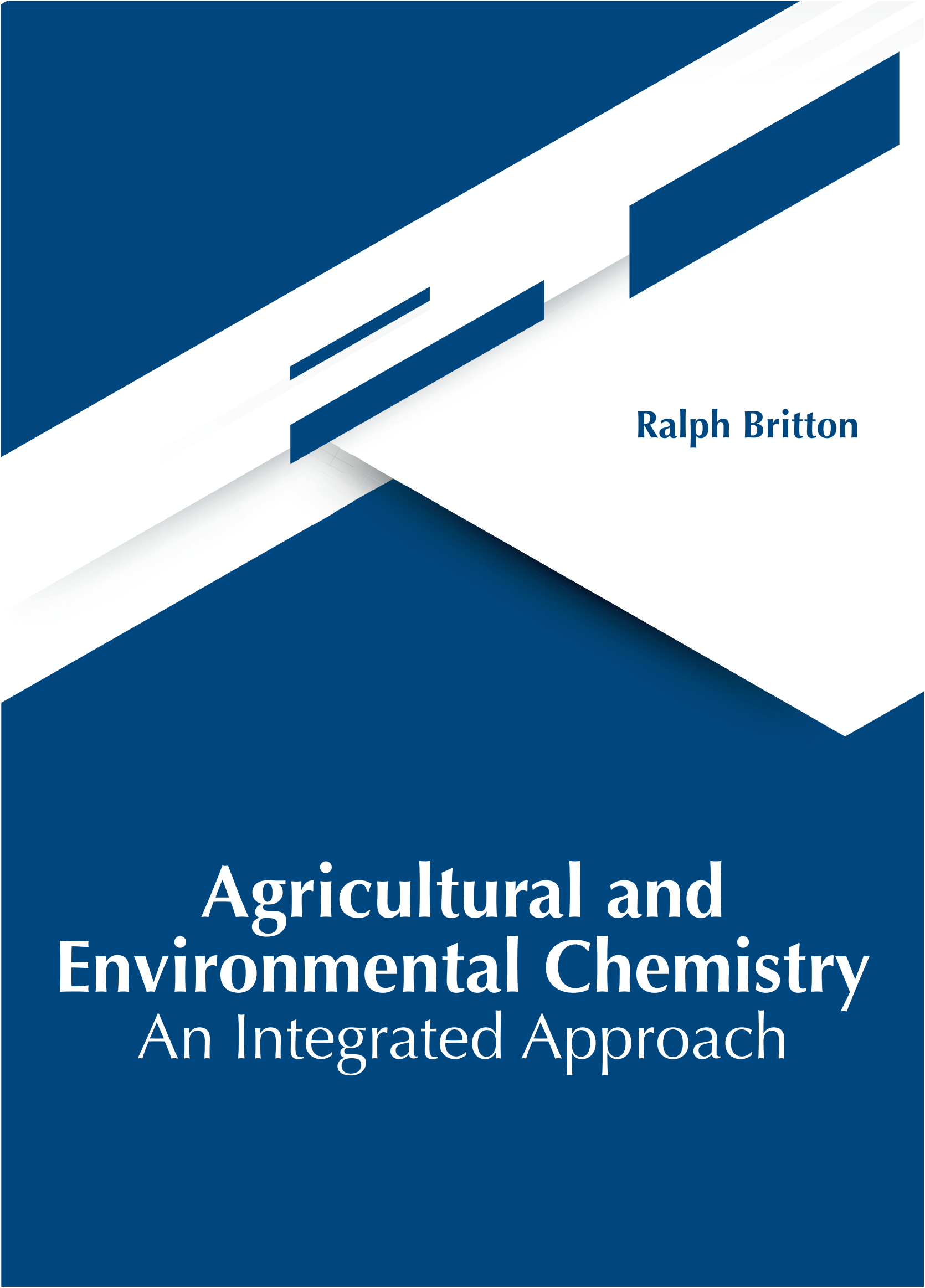




Ralph Britton

Agricultural and Environmental Chemistry

An Integrated Approach

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Agricultural and Environmental Chemistry: An Integrated Approach

Agricultural and Environmental Chemistry: An Integrated Approach

Edited by **Ralph Britton**

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Edited by Ralph Britton

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Preface

The world is advancing at a fast pace like never before. Therefore, the need is to keep up with the latest developments. This book was an idea that came to fruition when the specialists in the area realized the need to coordinate together and document essential themes in the subject. That's when I was requested to be the editor. Editing this book has been an honour as it brings together diverse authors researching on different streams of the field. The book collates essential materials contributed by veterans in the area which can be utilized by students and researchers alike.

This book provides detailed information about the fields of agricultural and environmental chemistry. Agricultural chemistry deals with all the aspects of agricultural production, processing of raw materials, environmental monitoring and evaluation whereas, environmental chemistry studies the chemical and biochemical phenomena occurring in nature. Agricultural chemistry often aims at improving the fertility of the soil which is a major issue in the present times. On the other hand, environmental chemistry chiefly explains the effect of human activities on soil. Both these fields are closely associated and often contribute to the research and progress of each other. They have become crucial in today's context due to rapidly changing environmental patterns. The exclusive studies included in this book provide an in-depth knowledge of the developments in this field. It is a valuable source of information for professionals and students involved in this area of science.

Each chapter is a sole-standing publication that reflects each author's interpretation. Thus, the book displays a multi-faceted picture of our current understanding of applications and diverse aspects of the field. I would like to thank the contributors of this book and my family for their endless support.

Editor

Reducing the pollutant load of olive mill wastewater by photocatalytic membranes and monitoring the process using both tyrosinase biosensor and COD test

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Photocatalytic technique had already been employed in the treatment of olive mill wastewater (OMW) using the photocatalysis in suspension. The coupling of photocatalytic and membrane techniques should result in a very powerful process bringing great innovation to OMW depollution. Despite the potential advantages using these hybrid photoreactors, research on the combined use of photocatalysis and membranes has so far not been sufficiently developed. The present paper describes a study to assess the photocatalytic efficacy of a new ceramic membrane containing titanium dioxide, irradiated by UV light, used to abate the pollutant load of OMW. Good results were obtained (more than 90% of the phenol content was removed and the COD decrease was of the order of 46–51% in 24 h) particularly using the ceramic membrane compared with those offered by analogous catalytic membranes made of metallic or polymeric materials.

Keywords: olive mill wastewater (OMW), photocatalysis, photoreactor, tyrosinase biosensor, COD

INTRODUCTION

There are literature reports of a good number of studies (Saez et al., 1992; Paraskeva et al., 2004; Dhaouadia and Marrot, 2008) on the possibility of abating or reducing the pollutant load of olive mill wastewater (OMW). The olive oil extraction process generates large amounts of dark liquid effluent known as OMW, as high as 0.5–0.8 m³ per tonne of olives treated. This effluent consists of a mixture of "vegetation water" coming from the olives, and water added during oil extraction process. OMW is one of the most contaminated effluents. OMW is a foul smelling acidic wastewater, composed of water (83–92 W/W%), organic matter (4–16 W/W%) and minerals (1–2 W/W%). OMW disposal on farmland is the cause of serious problems owing to the wastewater's high phenol and polyphenol content.

Owing to their antibacterial effects, phenols and polyphenols are the most problematic compounds contained in the OMW (Saez et al., 1992; Pozzo et al., 1997). Alternative processes have been proposed to reduce pollutant problems such as those due to OMW. According to the literature, the proposed OMW treatment processes can be physical-chemical, biological or combined treatment (Boari and Mancini, 1990; Fiestas De Ursinos and Padilla, 1992; Pozzo et al., 1997; Paraskeva and Diamadopoulos, 2006). Treatment of OMW by advanced oxidation processes (AOPs), such as electrochemical oxidation, has recently been increasing (Andreozzi et al., 1999; Drouiche et al., 2004; Inan et al., 2004; Gotsi et al., 2005; Giannis et al., 2007). Recent studies used solar photocatalytic pilot plants to obtain the OMW degradation by combined TiO₂ (very widely used also in the degradation of textile reactive dye) and photo-Fenton catalysis system (Ahmadi et al., 2005). The present

research therefore aims at investigating the catalytic effect of titanium dioxide, together with the action of ultraviolet light and the oxidizing effect of hydrogen peroxide. The tests were performed in a photoreactor in which three different types of heterogeneous membrane containing titanium dioxide were successively inserted, one at a time. Of these three different membranes one was ceramic, one polymeric and the third metallic. Significant advances in membrane technology research aimed at extending membrane potential have recently been reported.

Numerous membrane applications have also been proposed that are regarded as economically competitive due to the availability of membranes with higher flux and lower process costs. In particular, ceramic membrane development has been one of the principal targets. These membranes have proved suitable for high temperature, corrosive and high pressure applications with good durability (DeFriend et al., 2003; Falamaki et al., 2004; Yoshino et al., 2005; Bouzerara et al., 2006; Wang et al., 2006; Nandi et al., 2008; Kim and Van der Bruggen, 2010). In the present research, aimed at monitoring the photocatalytic treatment of olive oil mill wastewater in a batch photoreactor containing a catalytic (TiO₂) membrane, an investigation was made of the principal parameters affecting the water treatment process, such as the variation in concentration between the beginning and the end of the photocatalytic process of compounds selected as indicators, i.e., polyphenols, or COD variation. In addition, an assessment was made of the amount of catalyst, the presence or absence of oxidizing agent and the varying power of the UV radiation used for photocatalytic irradiation by performing various tests on mill wastewater samples. The catalytic

process was monitored by periodically taking samples from the reactor and analyzing them in order to determine both the total polyphenol content using a tyrosinase enzymatic biosensor previously developed by the authors (Campanella et al., 2005, 2006, 2007), and the COD (Chemical Oxygen Demand), measured colorimetrically by the dichromate method (Rand et al., 1979).

EXPERIMENTAL SECTION

MATERIALS

Raw materials for ceramic membranes such as clay, kaolin, feldspar, quartz, calcium carbonate and sodium carbonate were provided by Imerys Minerali SPA, Avezzano, Italy. Polysulfone, PVDF (Polyvinylidene fluoride), 1-octanol and N-methyl-pyrrolidone (NMP) was purchased from Sigma Aldrich srl, Milan, Italy. The metallic membrane was supplied by SETEC srl, Civita Castellana, Italy. Titanium oxide (P25) from Evonik Degussa GmbH, Frankfurt am Main, Germany. COD was determined using test kits (LCI 400 CSB/COD/DCO9) supplied by Hach Lange, Dusseldorf, Germany. The tyrosinase enzyme for the biosensor from mushroom (2400 U mg^{-1}) (EC 1.14.18.1) and the dialysis membrane (D-9777) were supplied by Sigma, St. Louis, Mo, U.S.A. Potassium chloride, phenol, phosphate for the buffer, potassium dichromate and the other chemical reagents were of analytical reagent grade and supplied by Carlo Erba, Milan, Italy. Lastly, the Kappa-carrageenan was from Fluka AG, Buchs, Switzerland.

SAMPLES

The OMW samples were provided by a three-phase olive oil mill company located in Lazio, Italy. In **Table 1** the principal characteristic of the studied samples are summarized.

APPARATUS

A tyrosinase biosensor previously developed by the authors (Campanella et al., 2005, 2006, 2007), (see **Figure 1**) was used for Total Phenols (TPh) determination. The tyrosinase biosensor was assembled using an amperometric oxygen electrode by Universal Sensor Inc., New Orleans (U.S.A.), Mod. 4000-1, connected to a mod. 551 VA-Detector Amel potentiostat coupled to a Mitel MK 5001 Multimeter and to a mod. d5126-2 Omniscribe analog recorder. Total Chemical Oxygen Demand (Rand et al., 1979) was determined using a Hach DR 6000 Spectrophotometer (Hach Lange, Dusseldorf, Germany) and a LT 200 Thermostat for standard and special digestions. A batch type photoreactor designed for laboratory scale analysis was utilized. The bench-scale OMW experimental setup consisted of an aerated photoreactor and a side-stream tubular membrane module. The aerated bioreactor was made of glass and had an operating volume of 2 litres, a diameter of 10 cm and length of 55 cm. Air was introduced into the reactor using filtered in-house compressed air via air diffusers placed at the bottom of the reactor. UV irradiation was provided by a 450 W high pressure Hg lamp, emitting radiation in the 300–400 nm range, or with a 36 W low pressure lamp, emitting radiation in the 250–300 nm range, both produced by Helios Italquartz, Milan, Italy.

Table 1 | Properties of OMW samples used in this study.

COD, (g/L)	47
Total Phenols (TPh), (g/L)	8.1
pH	4.6
Color (absorbance at $\lambda = 450 \text{ nm}$)	Dark brown

Properties of OMW prior to dilution.

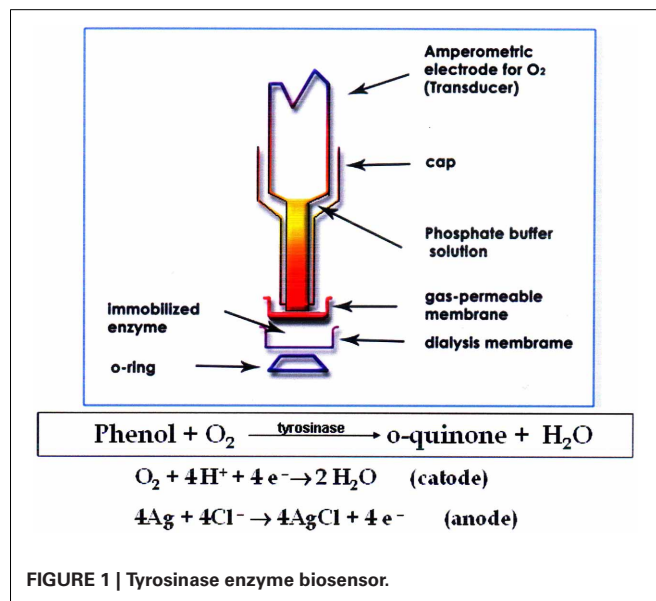


FIGURE 1 | Tyrosinase enzyme biosensor.

PHOTOCATALYSIS

Photocatalytic processes make use of a semiconductor metal oxide (TiO_2) as catalyst and H_2O_2 as oxidizing agent. The mechanism of photocatalytic oxidation of organic compounds is well known (Hoffmann et al., 1995; Linsebigler et al., 1995; Herrmann et al., 1999; Gelover et al., 2004; Chatterjee and Dasgupta, 2005; Fostier et al., 2008) and involve multiple processes. Initially, an electron-hole (e^-/h^+) pair is generated in the semiconductor particles (Hoffmann et al., 1995), when the surface is irradiated with energy (greater than) or equal to the band gap. Electrons are excited from the valence band (VB) to the conduction band (CB) of the semiconductor, thus creating an electron vacancy at the VB edge. The VB hole is strongly oxidizing, whereas the CB electron is strongly reducing. A hole can migrate to the surface and oxidize an electron donor; in turn, at the surface, the semiconductor can donate electrons to reduce an electron acceptor. Consequently, the semiconductor particle can act either as an electron donor or an electron acceptor for molecules in the surrounding medium, depending on the charge transfer to the adsorbed species (Linsebigler et al., 1995; Chatterjee and Dasgupta, 2005). Overall, the mechanism of photocatalysis can be divided into five steps: (1) transfer of reactants in the fluid phase to the surface; (2) adsorption of the reactants; (3) reaction in the adsorbed phase; (4) desorption of the products; and (5) removal of products from the interface region (Herrmann et al., 1999). A photocatalyst is a substance that, after being irradiated by light, can induce a chemical reaction in such a way that the actual substance of the catalyst will not be consumed (Gelover

et al., 2004). Among the various types of photocatalyst, TiO_2 is the most commonly used semiconductor photocatalyst in the conversion of organic pollutants into harmless substances (Fostier et al., 2008). It is known to be relatively inert, corrosion resistant, and less toxic and cheaper (Madhusudan Reddy et al., 2003) than other photocatalysts such as ZnS , WO_3 , etc. Several catalysts have been tested so far (Do et al., 1994; Sayama and Arakawa, 1997; Valden et al., 1998; In et al., 2007), although TiO_2 in the anatase form seems to have the most appropriate attributes such as high stability, good performance and low cost (Yin et al., 2001; Andersson et al., 2002).

THREE DIFFERENT CATALYTIC MEMBRANES (CERAMIC, POLYMERIC AND METALLIC), FABRICATION

Substrates cover a wide range of ceramic, polymeric, glass and metallic materials (particularly stainless steel).

Ceramic membrane preparation

For the fabrication of the ceramic membrane, capable of liquid waste treatment, several inorganic raw materials were used: clay (19%W/W), kaolin (33%W/W), feldspar (16%W/W), quartz (20%W/W) calcium carbonate (10%W/W), sodium carbonate (2%W/W). The various raw materials used in the fabrication of inorganic membranes had different functional attributes. Kaolin and clay gave the membrane low plasticity and high refractory properties. Sand contributed to the mechanical and thermal stability of the membrane. The porous texture of the ceramic was regulated by calcium carbonate which, under sintering conditions, dissociates into CaO and releases CO_2 gas. The path taken by the released gaseous CO_2 created the porous texture of the inorganic membrane and contributed to membrane porosity during the sintering process. Sodium carbonate acted as a colloidal agent and improved the dispersion properties of the inorganic precursors, thereby enhancing the homogeneity of the membrane structure. Likewise, feldspar acted as binder by creating silicate bonds among the elements to increase the mechanical strength of the ceramic membrane. The particle size distribution analysis of the ceramic body was checked using a Malvern Mastersizer 2000 laser granulometer during the membrane fabrication process. The data indicate that almost 90% of the particles had a diameter of less than $10\text{ }\mu\text{m}$. The average particle size of kaolin, calcium carbonate and quartz was 2.37, 4.11, and $8.40\text{ }\mu\text{m}$, respectively.

For fabricating this ceramic membrane these raw materials were mixed and a body slip prepared (Almandoza et al., 2004; Saffaj et al., 2004, 2006; Ersu and Ong, 2008; Palacio et al., 2009; Jeonghwan and Van der Bruggen, 2010). Lastly, the dry ceramic membrane was fired at 950°C . The paste was then cast over a gypsum mold of a circular cylinder (55 cm diameter and 5 cm thickness) (Chin et al., 2006). Subsequently, the ceramic body was de-molded and the circular cylinder dried at room temperature for 24 h. After that it was maintained at 100°C for 12 h in a hot air oven. Then the membrane was sintered for 5 h as follows: 1 h to attain 250°C , 3 h to attain 600°C , 1 h to attain 900°C and 1 h at a constant temperature of 900°C . During the transition from 100°C to 250°C , a low heating rate was maintained in order to eliminate the induction of thermal stresses caused by the loss of moisture. Subsequent cooling of the membrane was conducted by

an atmospheric cooling procedure adopted by switching off the muffle furnace that had previously been maintained at the established sintering temperature. After sintering, membranes had a hard, rigid and porous texture. Finally, the fabricated membrane was polished with silicon carbide abrasive paper (C-220) to obtain a membrane 53 cm in diameter and 4.5 cm thick. The internal surface of the cylinder was glazed with an enamel containing also 10% (w/w), or 30% (w/w), of TiO_2 , then fired again at 700°C . Physical characterization of the membrane was performed after firing. The open porosity of the membrane was evaluated using the water percentage absorption method. Chemical (i.e., corrosion) resistivity of the membrane was evaluated by subjecting the membrane to concentrated HCl and concentrated NaOH solution. Analysis of the membranes before and after the corrosion test was performed to detect any change in elemental composition. The mechanical strength of the membranes was tested using a three point bending load method (ASTM Standard C1211-02, 2003; JIS R 1601, 2008). All these quantitative tests were performed on the ceramic sample prepared for evaluating general membrane performance and characteristics.

Polymeric and metallic membrane preparation

To fabricate the polymeric membrane (Legrini et al., 1993; Molinari et al., 2001; Addamo et al., 2004; Cao et al., 2006; Damodar et al., 2009) a homogeneous polymeric solution was prepared containing a polymer, a solvent and an additive such as commercial polysulfone, 1-octanol and NMP, respectively, in addition a 10 or 30 percent by weight of TiO_2 . In practice the alcohol guaranteed a sufficiently fast phase separation in the water containing the coagulation bath. In order to achieve the desired structure of the membrane, PVDF (Polyvinylidene fluoride) with TiO_2 was used, as reported in literature (Byrne et al., 1998; Molinari et al., 2002). Casting solutions were prepared by mixing 10 wt.% polyvinylidene fluoride (PVDF), with different amounts of nanosize (20 nm) Degussa P25 TiO_2 catalyst particles (10 or 30 W/W%) in n-methyl-2-pyrrolidone (NMP) solvent at $60\text{--}65^\circ\text{C}$. The solution containing different percentages of TiO_2 was sprayed on to a PVC support. After the spraying step, the membrane was immediately exposed for 10 s to a (450 W) high pressure UV lamp to anchor the solution on the PVC support, which was then immersed in a $23\text{--}25^\circ\text{C}$ tap water coagulation bath for 1 day. Finally the PVDF/ TiO_2 composite membrane obtained was washed with distilled water.

Lastly, the metallic membrane was made by electro-depositing titanium (Ti) on the surface of a stainless steel foil support (Delplancke and Winand, 1988; Fernandez et al., 1995; Esplugas et al., 2002; Yanga et al., 2004). The stainless steel foil was cleaned by sonication in acetone and placed in a suspension of TiO_2 , Degussa P-25 (mainly anatase) in acetone (1 g in 100 mL). The suspension was first homogenized by sonication and stirred magnetically during the deposition. A counter-electrode of platinum was placed in the suspension just before the stainless steel substrate having the same size and shape. An appropriate potential was applied between the electrodes, the stainless steel foil acting as the cathode. Two samples were prepared by applying 200 V for 4 min. Due to their natural surface charge, the titanium particles moved to the stainless steel foil, thus forming a

layer. Finally, to improve adhesion, the sample was heated in a N_2 flow at 973 K for 4 h to produce the sample TiO_2 /steel. After anodic oxidation, the metallic membrane was rinsed with distilled water and dried in an oven at 40°C (Delplancke and Winand, 1988; Fernandez et al., 1995; Esplugas et al., 2002; Yanga et al., 2004).

OLIVE MILL WASTEWATER DEGRADATION PROCESS IN A PHOTOREACTOR

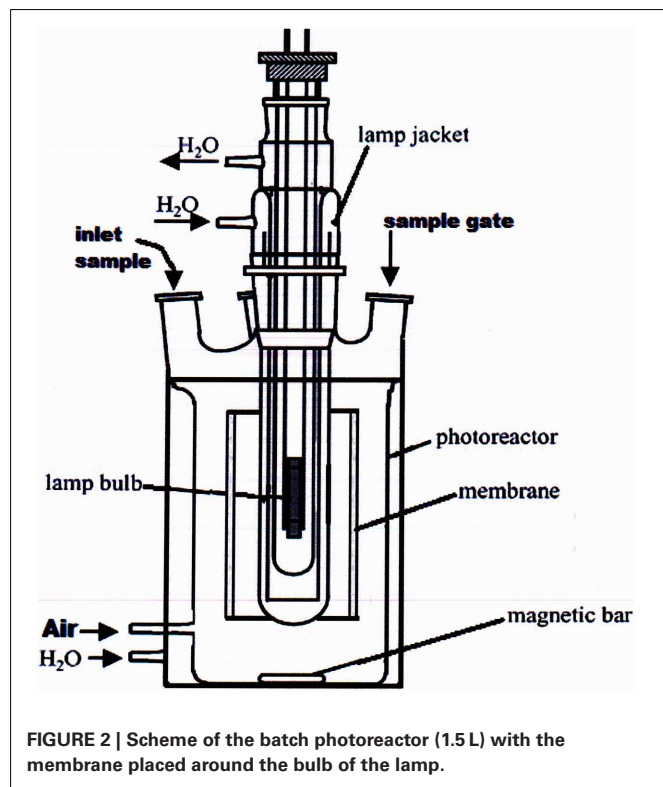
Experiments were conducted in a batch type laboratory scale photoreactor, as illustrated in **Figure 2**. The aerobic olive mill waste water photoreactor (AOP) consisted of a cylindrical vessel with a diameter of 8.0 cm. The working volume was 1.5 L, with provision made for UV irradiation and addition of hydrogen peroxide (the final concentration of H_2O_2 in the photoreactor was 5 mM). The catalytic ceramic membrane was mounted as required around the UV lamp, so that the TiO_2 present in the internal glazed surface was exposed to UV irradiation to achieve the degradation of pollutants. For the sake of comparison, in addition to this new type of ceramic membrane, also two other traditional membranes, either polymeric or metallic, were used alternatively in the same way. In a typical heterogeneous photocatalytic test, 300 mL of OMW were diluted 1:100 V/V with distilled water. Then 1.5 L of the diluted sample were placed in the reactor after adding 4 mL of H_2O_2 (0.20 M) in order to obtain a concentration of about 5 mM in the photoreactor and irradiated with a low or high pressure UV lamp. Periodically 5 mL of the sample was taken and their polyphenol content determined using a tyrosinase enzymatic biosensor, fabricated as described in previous papers (Campanella et al., 2005,

2006, 2007), while the COD was determined using the dichromate method (Rand et al., 1979).

RESULTS AND DISCUSSION

Some papers reported in literature describe the use of microporous membranes for waste water treatment (Di Serio et al., 2008; Cui et al., 2011; Athanasekou et al., 2012) coupled to the catalyst, and the immobilization of TiO_2 both physically deposited on the membrane surface, or confined in coating. In the present research our aim was to study the use of a ceramic membrane in which 10% W/W, or 30% W/W, TiO_2 was included in the glaze that cover the internal surface of the tubular membrane. This ceramic membrane had both a filtration and a support function. A new inorganic formulation was also tested for the fabrication of this ceramic membrane, which had an average particle diameter varying from 0.5 to 50 μm . In addition, the present research indicates that the ceramic membrane was fabricated with high contents of inexpensive kaolin (33%) and clay (19%). Thermal characterization suggested that the appropriate sintering temperature for the composition of materials selected is around 900°C. The membranes provided good mechanical strength (3 MPa flexural strength) and chemical stability (only 8% weight loss was found, if immersed in both acid or base media). The results obtained in all the tests performed using the UV lamp at high pressure, expressed in $mg L^{-1}$, are illustrated in the histograms in **Figures 3, 4**, while all the principal data obtained using UV lamp at low and high pressure, expressed in % removal of COD and phenols, are summarized in **Tables 2, 3** respectively.

Several tests were carried out involving the photodegradation process performed in the photoreactor described and the three different membranes were each used successively. In particular, the process was investigated both in the presence of H_2O_2 , but without any catalytic membrane, and with H_2O_2 and the catalytic membrane. The results obtained point to a moderate improvement due to the use of H_2O_2 in the photodegradation process as the percentage of COD removal increases from 28 to 29%, while the percentage polyphenol abatement varies from about 50 to 53%. Lastly the investigated process was carried out using two different UV lamps, one low and one high pressure. In particular, **Tables 2** and **3** show the percentage removal of COD and phenols with an UV lamp at low pressure (36 W) or at high pressure (450 W), respectively, after 24 h, in order to monitor the photocatalytic treatment of OMW in a batch photoreactor using a tyrosinase biosensor and COD test. Comparing the data in **Tables 2, 3** the results obtained show that the effect on the process trend does not change substantially whichever type of lamp is used. However, in optimal conditions, the percentage polyphenol abatement varies from about 93 to 97% and that of COD from about 52 to 56% on going from the low pressure lamp to the high pressure one. These results show that on going from the low pressure to the high pressure lamp, at constant treatment time, the pollutant load abatement does not vary by more than 5%, while the cost of the materials (high pressure lamps) and the energy consumption (in kWh) are much higher in the latter treatment. It is therefore more economical to use low pressure lamps in this treatment even though this means lower pollutant load abatement.



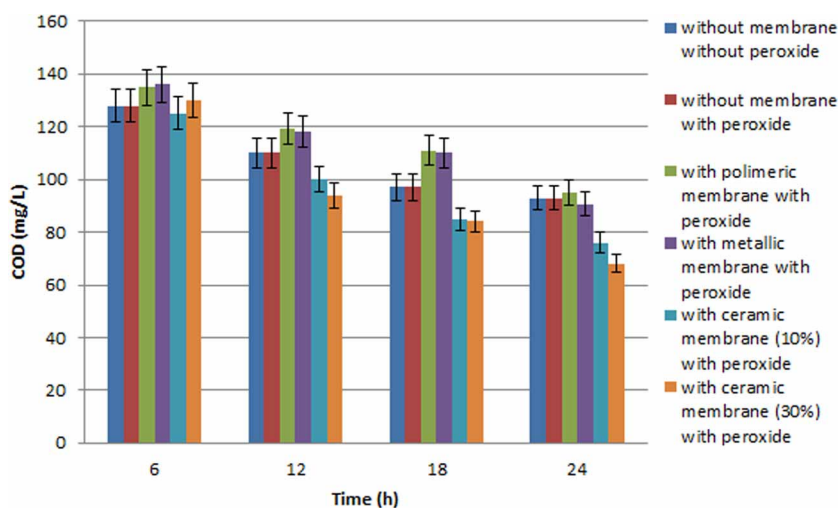


FIGURE 3 | Chemical oxygen demand variation during batch experiment with the three different membranes used with olive mill wastewater (diluted 1:100 with distilled water), performed with a (450 W) high pressure UV lamp.

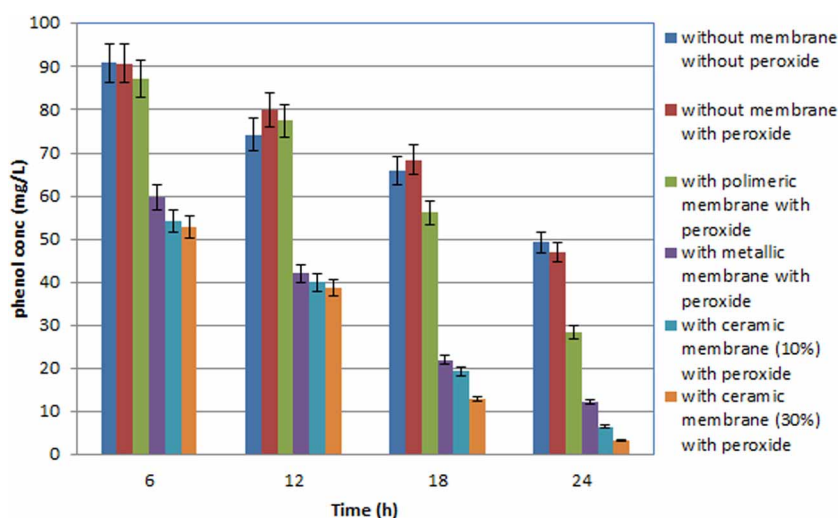


FIGURE 4 | Total phenolic compound variation in the OMW during batch experiment with the three different membranes used with olive mill wastewater (diluted 1:100 with distilled water), performed with a (450 W) high pressure UV lamp.

It was found that the reaction time have an important effect on COD and phenol removal (see the histograms behavior in **Figures 3, 4**). After 24 h and in the presence of TiO_2 , using the low pressure UV lamp, almost 46.0 and 89.1% of COD and phenols were, respectively, removed in this process involving the treatment of OMW using a ceramic membrane glazed with an enamel containing 10% W/W TiO_2 . The use of a metallic membrane, on which titanium dioxide, had been deposited using an electrolytic anodizing process, resulted in a percent COD abatement of 40.9% and a percent phenol reduction of 82.1%. Results also show that treatment efficiency increased with increasing TiO_2 concentration of about 30% in the ceramic membrane, achieving 52.4 and 93.3% in COD and TPh, respectively. This observation indicates that most of the biodegradable compounds initially present in the wastewater were destroyed or/and

less biodegradable intermediates were formed. Finally, in the case of the polymeric membrane the final COD removal was only of the order of 32.5% and that of phenols of the order of 64.2%.

The present research was carried out on suitably diluted OMW samples before photocatalytic treatment in the reactor (as described in the previous section). This allowed both the photodegradation process and the analytical methods employed to monitor pollutant load abatement during the process to be tested more accurately. In practice, the aim was above all to give priority to the study of the analytical aspects of the methods used. In future research, the next stage will be to repeat the same tests on increasingly less diluted samples of OMW up to the point of using non diluted "as is" samples, to investigate if and when the increased sample concentration ultimately has an effect on both

Table 2 | Percentage removal of COD and phenols with a (36 W) low pressure UV lamp after 24 h.

Experimental condition	% COD removal RSD% $\leq$ 5.0	% phenols removal measured with tyrosinase biosensor RSD% $\leq$ 5.0	pH of the solution after the process RSD% $\leq$ 5.0
Without membrane and without H ₂ O ₂	28.10	50.2	5.48
Without membrane and with H ₂ O ₂	29.05	53.8	5.85
With Metallic membrane and with H ₂ O ₂	40.85	82.1	6.95
With Polymeric membrane and with H ₂ O ₂	32.52	64.2	7.70
With Ceramic membrane (10 W/W% TiO ₂ on the surface) and with H ₂ O ₂	45.95	89.1	7.25
With Ceramic membrane (30 W/W% TiO ₂ on the surface) and with H ₂ O ₂	52.42	93.3	7.10

Table 3 | Percentage removal of COD and phenols with a (450 W) high pressure UV lamp after 24 h.

Experimental condition	% COD removal RSD% $\leq$ 5.0	% phenols removal measured with tyrosinase biosensor RSD% $\leq$ 5.0	pH of the solution after the process RSD% $\leq$ 5.0
Without membrane and without H ₂ O ₂	28.50	50.8	5.50
Without membrane and with H ₂ O ₂	29.15	53.8	5.95
With Metallic membrane and with H ₂ O ₂	44.85	87.1	6.85
With Polymeric membrane and with H ₂ O ₂	38.82	71.4	7.80
With Ceramic membrane (10 W/W% TiO ₂ on the surface) and with H ₂ O ₂	50.97	93.2	7.25
With Ceramic membrane (30 W/W% TiO ₂ on the surface) and with H ₂ O ₂	56.56	96.8	7.18

the efficiency of the photocatalytic process and the monitoring methods used themselves.

CONCLUSIONS

Data reported in the **Tables 2, 3** show how the new ceramic membrane containing TiO₂ seems to provide an excellent combination of thermal, mechanical and chemical stability in addition to good catalytic characteristics. Among the configurations described in this paper, the membrane photoreactor, which combines the advantages of both classical photoreactors and membrane processes, seems very promising. Photocatalytic degradation can be carried out reasonably quickly due to the high available irradiated surface area of the catalytic particles. This study shows that the ceramic membrane is a good candidate for the removal of pollutants in OMW. Indeed, the performance of polymeric, metallic and ceramic membranes was evaluated and compared as far as and the percentage removal of COD and total phenols was concerned. Results show that the ceramic membrane is very effective in removing both compared with the two other membranes. Lastly, it may be concluded that the use of the tyrosinase amperometric biosensor to monitor this process can be considered practical, useful and cheap in monitoring the photocatalytic process of mill wastewater.

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The hygroscopic behavior of plant fibers: a review

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Environmental concern has resulted in a renewed interest in bio-based materials. Among them, plant fibers are perceived as an environmentally friendly substitute to glass fibers for the reinforcement of composites, particularly in automotive engineering. Due to their wide availability, low cost, low density, high-specific mechanical properties, and eco-friendly image, they are increasingly being employed as reinforcements in polymer matrix composites. Indeed, their complex microstructure as a composite material makes plant fiber a really interesting and challenging subject to study. Research subjects about such fibers are abundant because there are always some issues to prevent their use at large scale (poor adhesion, variability, low thermal resistance, hydrophilic behavior). The choice of natural fibers rather than glass fibers as filler yields a change of the final properties of the composite. One of the most relevant differences between the two kinds of fiber is their response to humidity. Actually, glass fibers are considered as hydrophobic whereas plant fibers have a pronounced hydrophilic behavior. Composite materials are often submitted to variable climatic conditions during their lifetime, including unsteady hygroscopic conditions. However, in humid conditions, strong hydrophilic behavior of such reinforcing fibers leads to high level of moisture absorption in wet environments. This results in the structural modification of the fibers and an evolution of their mechanical properties together with the composites in which they are fitted in. Thereby, the understanding of these moisture absorption mechanisms as well as the influence of water on the final properties of these fibers and their composites is of great interest to get a better control of such new biomaterials. This is the topic of this review paper.

Keywords: natural fibers, composite materials, hydrophilic behaviors, ageing effects, durability

INTRODUCTION

Environmental concern has resulted in a renewed interest in bio-based materials. Among them, plant fibers are perceived as an environmentally friendly substitute to glass fibers for the reinforcement of composites, particularly in automotive engineering (Wambua et al., 2003; Suddell and Evans, 2005; Summerscales et al., 2010). Due to their wide availability, low cost, low density, high-specific mechanical properties, and eco-friendly image, they are increasingly being employed as reinforcements in polymer matrix composites (Bledzki and Gassan, 1999). In literature the term biocomposite is often used to define a polymeric matrix reinforced by natural fibers. The increasing number of publications during last 10 years including reviews, reflect the growing importance of these new biocomposites (Bledzki and Gassan, 1999; Mohanty et al., 2000; John and Thomas, 2008; Satyanarayana et al., 2009; Summerscales et al., 2010; Faruk et al., 2012). Indeed, their complex microstructure as a composite material makes plant fiber a really interesting and challenging subject to study. Research subjects about such fibers are abundant because there are always some issues to prevent their use at large scale (poor adhesion, variability, low thermal resistance, hydrophilic behavior). The choice of natural fibers rather than glass fibers as filler yields a change of the final properties of the composite. One of the most relevant differences between the two kinds of fiber is their response to humidity. Actually, glass fibers are considered as hydrophobic whereas plant fibers

have a pronounced hydrophilic behavior. Composite materials are often submitted to variable climatic conditions during their lifetime, including unsteady hygroscopic conditions. However, in humid conditions, strong hydrophilic behavior of such reinforcing fibers leads to high level of moisture absorption in wet environments (Céline et al., 2013). This results in the structural modification of the fibers and an evolution of their mechanical properties together with the composites in which they are fitted in (Dhakal et al. (2007); Symington et al. (2009); Placet et al. (2012b)). Thereby, the understanding of these moisture absorption mechanisms as well as the influence of water on the final properties of these fibers and their composites is of great interest to get a better control of such new biomaterials. This is the topic of this review paper.

ABOUT PLANT FIBERS

ORIGIN OF PLANT FIBERS

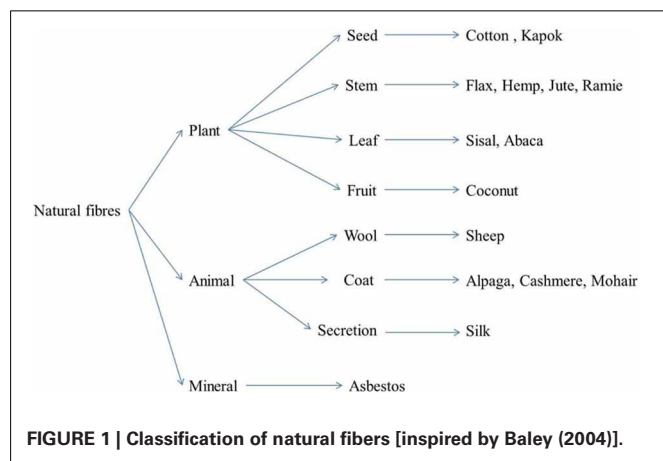
In nature, there is a wide range of natural fibers which can be distinguished by their origin. Precisely, natural fibers are divided into three categories including animal fibers, mineral fibers, and plant fibers (Figure 1). In the present paper we will focus on this last group. For details about the other kind of fibers, interesting readers could refer to (Speil and Leineweber, 1969; Champness et al., 1976; Blicblau et al., 1997; Fu et al., 2009). As can be seen in Figure 1, plant fibers could also be classified according to their location in the plant. For example, bast fibers as flax, hemp or

jute (Mohanty and Misra, 1995; Summerscales et al., 2010) are extracted from the stem of the plant whereas other fibers could be extracted from seeds (cotton) (Chand et al., 1988), fruit (coconut, pineapple), (Arib et al., 2004) or even the leaves of the plant (sisal) (Mukherjee and Satyanarayana, 1984; Li et al., 2000). The origins and properties of these different fibers have been described in a detailed review paper by (Faruk et al., 2012). Vegetal fibers are extracted from the plant using widely known and controlled processes in the textile industry. Some authors studied the influence of growth conditions and extraction processes on their properties (Keller et al., 2001; Coroller et al., 2013; Martin et al., 2013). As an example in Martin et al. (2013) works, moisture sorption and mechanical properties of flax fibers were found to be dependent upon the degree of retting.

CHEMICAL AND STRUCTURAL ORGANIZATION

Chemical composition

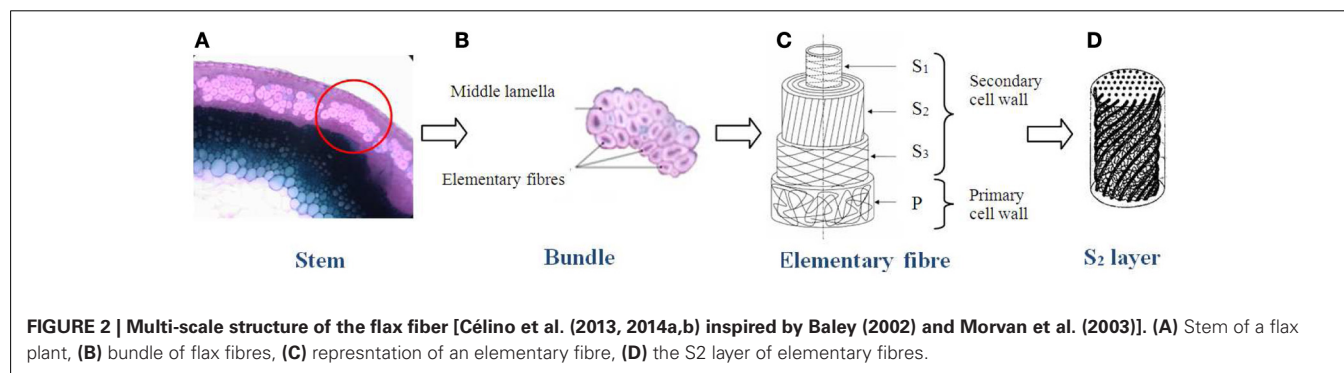
Plant fibers are mainly composed by sugar based polymers (cellulose, hemicelluloses) combined with lignin and pectin. Additional components, such as wax or oil could be found as well as structural water (De Rosa et al., 2010). Climatic conditions, age or degradation process influence the chemical composition which varies from plant to plant and within different part of a same plant. In their literature review (Faruk et al., 2012) listed the range of the average chemical constituent for a wide variety of plant type.



Cellulose is the major constituent of such fibers. It is a linear polymer chain consisting of D-glucopyranose units joined together by β -1,4-glycosidic linkages. Hydrogen bonds between the different macromolecules give the assembly various interesting physical properties, including the ability to form crystalline structures. As a result, cellulose has a semi crystalline form: there are both highly crystalline regions and amorphous regions. Crystalline cellulose displays six different polymorphs with the possibility of conversion from one form to another. The cellulose I crystal form, or native cellulose, also comprises two allomorphs, namely cellulose I α and I β (Sugiyama et al., 1991). The ratio of these allomorphs is found to vary from plant to plant. In bast fibers as flax, jute or hemp, cellulose I β is found to be predominant (Sarko and Muggli, 1974; Nishiyama et al., 2002). Crystalline regions are called crystallites. The threadlike entity which arises from the linear association of these components is called the microfibril; it forms the basic structural unit of the plant cell wall. These microfibrils are composed by several thousands of cellulose chains. Their diameter can be measured by X-ray diffraction. It is in the nanometer range, between 5 and 30 nm, depending on the authors and the type of fiber (Näslund et al., 1988; Fink et al., 1990; Eichhorn et al., 2001; Astley and Donald, 2003). In the longitudinal direction, the Young's modulus of these microfibrils is about 137 GPa (Sakurada et al., 1962). These features provide their good mechanical properties to plant fibers. In most natural fibers, the microfibrils orient themselves at an angle to the fiber axis called the microfibril angle. This angle has a significant influence on the mechanical properties of the fiber. The lower it is, better the properties are (Bourmaud et al., 2013). Cave developed a technique to measure it by using X-ray diffraction (Cave, 1997). It varies from plant to plant. Resources on cellulose can be found at references (Eichhorn et al., 2001; Heinze and Fischer, 2005).

Structural organization

Plant fibers have a multi-scale structure and they can be used at different scales for composite materials reinforcement (**Figure 2**). Indeed, fibers could be conditioned as fabric yarn (Madsen and Lilholt, 2003), bundle of fiber or even unit fibers (Baley, 2002; Placet, 2009). A bundle of fibers (**Figure 2B**), is a gathering of several elementary fibers, linked together by a ten micrometers wall mainly composed of pectin and lignin. This wall is called middle lamella (Morvan et al., 2003). Plant fiber yarns consist of a large number of relatively short plant fibers that are twisted with



an angle to the yarn axis in order to provide axial strength to the yarn (Madsen et al., 2007). The unit fibers have a multi cell wall structure (**Figure 2C**). The section is polygonal but usually assumed as circular for the calculation of mechanical properties. Basically, it is represented by a hollow polyhedron, decomposed into several walls and layers. The external wall is called the primary wall. It presents a relatively small thickness compared to the total thickness of the fiber. This wall is essentially composed of pectin, low crystalline cellulose, hemicellulose and waxes in a lower amount (Gorshkova et al., 2000; Zykwincka et al., 2008). The secondary wall, which represents about 90% of the total section, is divided into three layers. It is mainly composed of cellulose microfibrils oriented parallel to each other and embedded in an amorphous matrix composed of hemicellulose, pectin, and lignin. The three layers are different from each other because of their different thickness and structural organization (microfibril angle, chemical composition). The thickest layer is the S2 layer. It represents 70–80% of the secondary wall thickness. Thus, the fiber properties are largely governed by the feature of this layer.

In first approximation, the unit fibers can be considered as composite materials with an amorphous matrix of hemicellulose and reinforced by cellulose microfibrils which are oriented in parallel and form a helix angle with the axis of the fiber. In the S2 layer, this angle is about 10° (**Figure 2D**). In the other layers, the microfibrils are not oriented at the same angles as shown in the schematic representation of Baley (2002). The hollowed part is called the lumen. It gives the fibers a tubular structure.

PHYSICAL AND MECHANICAL PROPERTIES

As mentioned before, plant fibers have the properties to compete with glass fibers as reinforcement for composite materials. Because of their low density, they have good specific mechanical properties, particularly concerning their stiffness. **Table 1** presents the important mechanical properties of commonly used fibers (Oksman et al., 2003; Satyanarayana and Wypych, 2007; Bodros and Baley, 2008; Ochi, 2008; Summerscales et al., 2010; Bourmaud, 2011; Faruk et al., 2012).

Table 1 | Mechanical properties of different fibers (Oksman et al., 2003; Bodros and Baley, 2008; Ochi, 2008; Satyanarayana et al., 2009; Summerscales et al., 2010; Bourmaud, 2011; Faruk et al., 2012).

Fiber	Density	Young's modulus (GPa)	Tensile strength (MPa)	Elongation at break (%)
Flax	1.54	27.5–85	345–2000	1–4
Ramie	1.5–1.56	27–128	400–1000	1.2–3.8
Hemp	1.47	17–70	368–800	1.6
Jute	1.44	10–30	393–773	1.5–1.8
Sisal	1.45–1.5	9–22	350–700	2–7
Coconut	1.15	4–6	131–175	15–40
Cotton	1.5–1.6	5.5–12.6	287–597	7–8
Nettle	1.51	24.5–87	560–1600	2.1–2.5
Kenaf	1.2	14–53	240–930	1.6
Bamboo	0.6–1.1	11–17	140–230	–
E-glass	2.5	70	2000–3500	2.5
Carbone	1.4	230–240	4000	1.4–1.8

As seen in **Table 1**, the properties of plant fibers may differ for a given fiber. In fact, the major problem, with such fiber is the high variability of their properties. Thus, in literature, there is a large amount of data showing relatively wide distribution. First, this variability can be explained by differences in the fibers chemical composition and structure (microfibrillar angle, crystallinity, defects) due to the environmental conditions during the growth (Bourmaud et al., 2013). Secondly, it can be explained by different testing methods employed or different environmental conditions (relative humidity, temperature, speed loading, number of sample tested) (Placet et al., 2012a). Moreover, as mentioned before, plant fibers can be investigated at different scales (fiber bundles or unit fibers). In the literature, there are mechanical data from both fibers bundles (Madsen et al., 2007; Charlet et al., 2011) and elementary fibers (Baley, 2002; Placet et al., 2012b). When testing is performed at the bundle scale, there are slippage effects of the fibers relative to each other in the middle lamella. Thus, generally, the properties of fiber bundles are lower than those of the elementary fibers. Charlet et al. (2011) studied the mechanical behavior of the middle lamella of flax fiber bundles. Authors showed low shear strength of this interface which can explain the weaker mechanical properties of bundle.

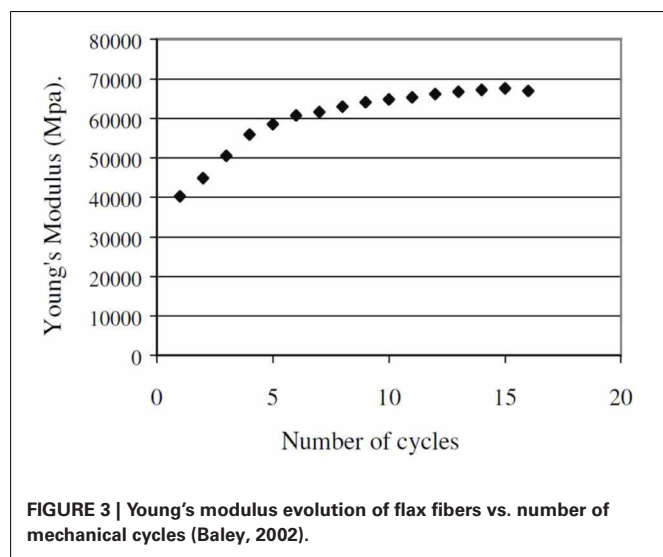
As mentioned in Structural organization, a plant fiber is a composite made of three polymers (cellulose, hemicellulose, and lignin), in which the unidirectional cellulose microfibrils constitute the reinforcing elements in the matrix blend of hemicellulose and lignin. This structure could be built as multi-ply construction with layers P, S1, S2, and S3 of cellulose microfibrils presenting different angles to the fiber axis (**Figure 2C**). Then the elastic properties could be calculated using classical laminated theory (Gassan et al., 2001). Transition scales models should also be used to predict hygro-elastic properties of such fibers. Mori and Tanaka models for example should be developed as described in Fréour et al. (2006). In order to take into account the disorientation of microfibrils reinforcement, methods presented by Lacoste et al. (2010, 2011) should be used. Mechanical properties of these three polymers have been widely studied in literature. Tashiro and Kobayashi (1991) and Gillis (1969) for example, presented data about cellulose. Moreover, Cousins, in the 80s, helped to build a valuable database for the lignin and hemicellulose properties (Cousins et al., 1975; Cousins, 1976, 1978). Because of its specific structure, plant fibers have an anisotropic behavior. In the longitudinal direction, they have good mechanical properties via microfibrils whereas in the radial direction mechanical properties are lower and more variable because of the amorphous blend properties.

Plant fibers show a specific behavior under mechanical cycles. Baley (2002) was the first to show that the Young's modulus of flax fibers increases with increasing the number of cycles (**Figure 3**). Moreover, a plastic deformation appears after the first cycle. To explain these results, the hypothesis of a new arrangement of the microfibrils in the fiber with an increase of the crystallinity degree has been proposed by the author. Then, the more the microfibrils are aligned with the fiber axis, the better will be the mechanical properties in this direction.

A similar effect has been shown by Placet et al. (2012b) for hydrated hemp fibers. Crystallization of plant fibers under tensile test has been highlighted by Astley and Donald (2003) using the X-ray diffraction. Reorientation of microfibrils during the tensile test has also been confirmed by various studies (Keckes et al., 2003; Burgert, 2006; Placet et al., 2011).

The mechanical performance of plant fibers are influenced by different parameters including: the cellulose content, the microfibrillar angle, the fiber diameter, the temperature, the presence of defects and the water content inside fibers. The latter case will be the purpose of a next section.

As cellulose is the stiffer component of natural fibers, the higher the cellulose content is, the better will be the mechanical properties. The microfibrillar angle has also a major influence on the elastic properties of the plant fibers. In fact, the weaker is this angle, better are the properties because plant fibers behave as a composite material which presents better mechanical properties in the reinforcement direction. Regarding the influence of diameter, most studies conducted on plant fibers in traction showed that both the Young's modulus and tensile strength increased when the diameter of the tested fibers decreased (Baley, 2002; Andersons et al., 2005; Charlet et al., 2010; Duval et al., 2011). Recently, Placet et al. (2012a) found out the causes of this dependence while studying hemp fibers. Using a mathematical model and reconstructing a 3D image of the fibers, they showed that their Young's modulus is dependent primarily on the size of the lumen and secondly on the diameter of the fiber outer layer. The temperature also affects significantly the mechanical properties of such fibers. It can lead to the emergence of defects resulting in a decrease of the overall mechanical properties of the fibers (Gassan et al., 2001; Stamboulis et al., 2001). The occurrence of defects in such materials is also a source of variability of the plant fibers mechanical properties. These defects can appear during the different extraction and processing steps of the fibers and especially during the stage of retting (Bourmaud, 2011). The influence of all these parameters has been studied in details by Mukherjee and Satyanarayana (1986).



SCIENTIFIC OBSTACLES TO THEIR EFFICIENT USE AS REINFORCEMENT IN COMPOSITE MATERIALS (RESEARCH TOPICS ON NATURAL FIBERS)

Table 2 summarizes the advantages and drawbacks of these fibers. In fact, nowadays, there are some issues that prevent their use at a large scale, in composite materials. These different points constitute interesting research works.

One of the main disadvantages related to the use of natural fibers as reinforcement in composites is the poor adhesion between fiber and matrix. In composites, the matrix acts as a binder to transfer fibers stiffness in the material. If its adhesion with the fibers is weak, the composite will not have desired properties. In addition, it will be vulnerable to the environment in which it will be used and its lifetime should be shortened. A lot of researches are conducted to improve the adhesion of the fibers with polymeric matrix by modifying the fiber surface. Two approaches are proposed by the authors: physical treatments (plasma, corona treatment...) or chemical modification (maleic anhydride, organosilanes, isocyanates, sodium hydroxide, permanganate, and peroxide...) (Gauthier et al., 1998; Hill et al., 1998; Gassan and Bledzki, 1999; Tripathy et al., 1999; Mishra et al., 2000; Mohanty et al., 2000; Bessadok et al., 2007; Islam et al., 2010; Alix et al., 2011, 2012). Unfortunately, the treatments proposed in the literature don't always make it possible to keep the integrity of the fibers, as well as their natural character.

Another disadvantage of such fibers is the variability of their properties depending on the batch, the variety and even the location of the fiber in the plant. For example, comparing the mechanical properties of flax fibers, located at different positions in the stem (Charlet et al., 2007) showed that flax fibers located in the center had better mechanical properties than the others.

The low temperature resistance of these fibers constitutes another drawback. Thus, the process temperature of the composite in which they are fitted should not exceed 200°C. Beyond this temperature, the fiber integrity is not guaranteed. The use of natural fibers implies a restriction about the choice of the matrix.

The resistance of such fibers to fungus can also raise some problems (storage conditions, process conditions, use in humid conditions).

Finally, the hydrophilic nature of fibers is a major problem for their use as reinforcement in polymers. In fact, it has been showed that the absorption of water by the plant fibers results in a decrease of the composite performances in which they play the role of reinforcement (Rangaraj and Smith, 2000). Research

Table 2 | Advantages and drawbacks of plant fibers.

Advantages	Disadvantages
Low cost	Hydrophilic behavior
Recyclable	Dimensional instability
Zero fingerprint CO ₂	Low thermal resistance
← biodegradability →	
Renewable resources	Variability
Low density	Anisotropic behavior
High specific mechanical properties	Discontinuous
Good thermal and acoustics isolation	
Non abrasive	

has to be carried out to understand absorption mechanisms in such fibers. The following section is a literature review about their hydrophilic behavior as well as the influence of water on their properties.

HYDROPHILIC BEHAVIORS OF NATURAL FIBERS

For their use as reinforcement, the hydrophilic nature of plant fibers has to be considered with carefulness for several reasons. First, during the life cycle of the material, water absorption could induce a volume change of the fibers inside the composite, leading to the development of internal stresses. On the other hand, during the polymerization process of the matrix above 100°C, a vaporization of water trapped inside fibers could occur, leading to their shrinkage. These swelling and shrinkage of the fibers surrounded by the matrix generate internal stresses at the fiber/matrix interface and can eventually lead to the damage of the latter and to a significant degradation of the initial properties of the composite. The works of Rangaraj and Smith (2000); Dhakal et al. (2007); Le Duigou et al. (2009); Hu et al. (2010); Assarar et al. (2011) deal with water sorption of composites reinforced by bio-based fibers. For example, in their work on the water uptake of a flax fiber composite material (Assarar et al., 2011) showed an increase of the water content absorbed, compared to a material consisting of the same matrix reinforced with glass fibers. Le Duigou et al. (2009) studied the behavior of a composite PLLA/flax in immersion in seawater. The weight gain curves showed the influence of the cellulose fibers. The saturated moisture contents of the specimens were around 5.6%. As a consequence, adding flax fibers has resulted in a 17-fold increase in weight gain compared to unreinforced PLLA. The apparent diffusion coefficient was also significantly higher. Similar results were advanced by Lee and Wang (2006); Chow et al. (2007); Alix et al. (2011). Secondly, Le Duigou et al. (2009) showed a loss of the mechanical properties of the composite and an irreversible damage of the fiber/matrix interface during wet ageing. As mentioned before, this interface is a critical area considering the moisture absorption. The water diffusing in the composite material creates hydrogen bonds with the fibers, which can lead to the reduction of the interactions between the fibers and the matrix. Dhakal et al. (2007) showed an increase in moisture absorption with the volume fraction of fiber, for composite polyester/hemp immersed in water at 25°C. The relationship between volume fraction of fiber and water content was also clearly shown by George et al. (1998). In their work, Dhakal et al. (2007) showed a loss of mechanical properties in

bending with the amount of water absorbed. According to them, the moisture absorption leads to swelling of the fiber, resulting in the occurrence of micro cracks in the matrix. Then, as the composite cracks and gets damaged, capillarity and transport via micro cracks become active. The capillarity mechanism could involve the flow of water molecules along fiber/matrix interfaces as well as a process of diffusion through the bulk matrix. This could result in a debonding of the fiber and the matrix as shown on **Figure 4**. Eventually, those micro cracks create swelling stresses leading to the composite failure (Bismarck et al., 2002). Debonding effect and micro cracks were also observed in the case of other bio-based composites (Chow et al., 2007; Hu et al., 2010). Concerning the evolution of the stress at break of biocomposites, there are some inconsistencies in literature. On the one hand, Dhakal et al. (2007) show the tensile stress of hemp fiber reinforced unsaturated polyester composites increases about 22% after water immersion. On the other hand, Assarar et al. (2011) show a slight decrease of the tensile stress of flax/epoxy composites after immersion (about 5%). Such a trend is not completely consistent with (Le Duigou et al., 2009) works which present a large decrease of the stress at break (50%) when flax/PLLA composites were immersed in sea water.

Finally these studies highlight the following main points:

- A significant influence of the hydrophilic behavior of cellulose fibers on the maximum moisture absorption capacity of the composite they reinforce.
- An early damage of these kinds of composites, due to swelling and shrinkage effect of fibers.

In order to study the durability of such composites materials, it is thus relevant to understand water interactions occurring in plant fibers alone. In the following sections, the link between the microstructure of the fibers and their hydrophilic behavior will be studied and the influence of water on their properties will be investigated through a review of the literature.

LINK BETWEEN MICROSTRUCTURE AND HYDROPHILIC BEHAVIOR

The hydrophilic behavior of plant fibers, is mainly due to two factors: their composition and their specific structure. Generally, one of the most important factors controlling the water diffusion phenomenon in polymeric materials is the molecular interaction occurring between the diffusing compound and the substrate. The diffusion phenomenon is subjected to the ability of the

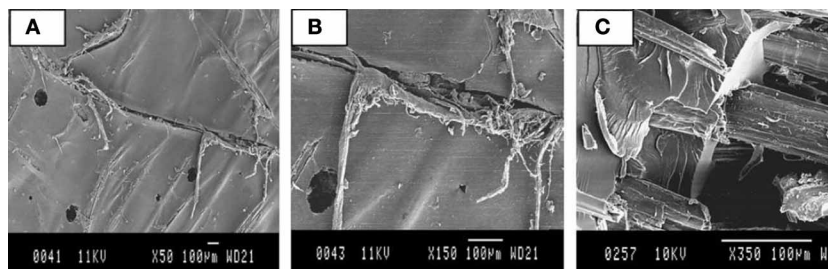


FIGURE 4 | (A) Matrix cracking, (B) Fracture running along the interface, (C) Fiber/matrix debonding due to attack by water molecules (Dhakal et al., 2007).

polymer molecular sites to establish hydrogen bonds with the water molecules. In plant fibers, components which have polar groups and thus are responsible for absorbing moisture are cellulose, hemicellulose, pectin and lignin (Berthold et al., 1998; Céline et al., 2014a).

Some authors have studied the water absorption in cellulose (Magne et al., 1947; Nelson, 1977). They showed that the water absorbed by the cellulose has very different properties from the free water. In their works, Nakamura et al. (1981) showed a significant decrease of bound water fraction in the cellulose as the crystallinity degree of the cellulose increases, by using a differential scanning calorimetry (DSC) technique. They also revealed that water molecules bind to the 3 hydroxyl of the glycosidic units of the amorphous phase while the absorption of hydroxyl sites on the crystalline phase is unpredictable. Based on these findings, it would appear that the moisture diffusion in the cellulose takes place mainly in the amorphous phase. Thus, most of the models used in the literature to describe the hygro-elastic behavior of plant fibers consider cellulose microfibrils to be 100% crystalline and then insensitive to moisture absorption (Neagu and Gamstedt, 2007; Marklund and Varna, 2009).

According to Davies and Bruce (1998), hemicelluloses which constitute the major part of the amorphous phase in plant fibers play an important role in the storage of moisture. This hypothesis is confirmed by the results of Pejic et al. (2008), who observed a significant decrease of the saturated weight gain of hemp fibers after removal of hemicellulose and lignin. In addition, Cousins (1976, 1978) showed that their mechanical properties significantly decreased with the moisture absorption.

Pectin, located in the middle lamella and the S1 layer are composed of highly polar carboxyl functions. These groups have the ability to create hydrogen bonds with polar solvents such as water. Depending on the retting rate of the fibers, their content varies (Martin et al., 2013). So, when fiber bundles are subjected to a humid environment, moisture uptake is more important than in the case of a single fiber, as the middle lamella mainly composed by pectin is a preferential area for water absorption.

Another factor determining the high level of moisture absorption in these fibers is their particular structure. These fibers are porous and have a high exchange surface. Thus, when the fiber is subjected to a humid environment, water can be stored inside the free volume of the structure. Currently, the porosity content in plant fibers is an unknown data.

To sum up, the diffusion of water is influenced by the fiber structure at different scales. At the unit fiber scale, the fiber exhibits a complex multi cell wall structure. This structure can in first approximation be assumed to behave similarly to its thicker layer S2 which usually constitutes more than 80% of the total diameter (Gorshkova et al., 2000). Actually, this layer is assumed to be a composite material with an amorphous phase (matrix) reinforced by a rigid crystalline phase (cellulose microfibrils) (Hearle, 1963). At this scale, diffusion of water would take place in the amorphous region. Besides, these regions are mainly composed by hydrophilic polymers (hemicelluloses and lignin). At the bundle scale, diffusion is privileged through the interface between fibers. This interface is called middle lamella. According to Morvan et al. (2003) the middle lamella is principally composed

of pectin where the carboxyl functions make easier the absorption of water by hydrogen bonding. The last structural factor influencing diffusion is the general porous structure of natural fibers. Water could be trapped inside pores.

SORPTION MECHANISMS

The precise mechanisms governing the transport of water in these fibers are still uncertain. The moisture absorption in these bio-based materials could be due to both diffusion phenomena and the effects of capillarity.

According to Bessadok et al. (2007), at high relative humidity when the water concentration exceeds a certain threshold, there is a relaxation of the existing voids in the structure, which leads to a significant swelling of the material. In fact, it seems that water enters in the fibers and breaks the secondary bonds between the macromolecules of cellulose. Then, water molecules could link to the network via hydrogen bonds resulting in a swelling of the material (Pejic et al., 2008). Hatakeyama and Hatakeyama (1998) studied the interaction of water with hydrophilic polymers. They showed that the water was more or less linked to the network of the material, highlighting the presence of bound and free water within such structures. The amount of bound water depends on the chemical structure of the material. This water is bound to the network by hydrogen bonds, breaking the existing bonds between the hydroxyl groups of the polymer chain.

Techniques for quantification and visualization of bound and free water are: DSC, Nuclear Magnetic Resonance (NMR), Raman spectroscopy and infrared spectroscopy (Hatakeyama et al., 2012). In NMR, it is possible to characterize different types of water, the molecular motion of the bound water and the water interactions with specific polymeric chain of the material in which they are inserted (Popineau et al., 2005). Using DSC, Nakamura et al. (1981) visualized and quantified these two types of water in cellulose samples. According to their works, there are actually three types of water called as follows: “freezing water,” “freezing bound water,” and “non-freezing water.” The first term refers to free water while the two others are respectively, water weakly and strongly linked to the network. The amount of “non-freezing water” is directly related to the molecular structure of the material. Fourier Transform Infra-Red spectroscopy (FTIR) has also been proved to be well adapted to study water sorption phenomenon since it allows characterizing molecular interactions involving potential sorption sites for water. In literature, FTIR spectroscopy has been widely used to study water transport in polymers and particularly to study the water sorbed into epoxy resins (Fieldson and Barbari, 1993; Cotugno et al., 2001; Feng et al., 2004). Recently Céline et al. (2014a) studied the water sorption on three natural fibers (flax, hemp, and sisal) using Fourier Transformed InfraRed spectroscopy. The spectral information enabled both qualitative and quantitative analysis of the moisture absorption mechanisms. The main chemical functions involved in the water sorption phenomenon were identified (**Figure 5**) and the absolute water content of the fibers was determined by using a Partial Least Square Regression (PLS-R) approach. Moreover, diffusion kinetics were plotted using this technique. More detailed analysis (by deconvolution of the OH valency band for example) could lead to the quantitative determination of the free and bound

water proportions, as described in different previous works on polymer-water mixtures (Cotugno et al., 2005; Mensitieri et al., 2006).

Concerning the diffusion kinetics, most of the authors historically used a classical Fick model to represent the diffusive behavior of such fibers subjected to hydrothermal ageing (Gouanvé et al., 2007; Bessadok et al., 2009). Recently, other authors proposed using the Parallel Exponential Kinetics model (PEK) to analyze the absorption and desorption curves of different cellulose fibers (Hill and Xie, 2011; Xie et al., 2011). They suppose that the diffusion process is limited by the swelling of the material and not by the diffusion phenomenon. This model represented by a double exponential, divides the diffusion kinetics into two first-order kinetics: a slow kinetics and a rapid kinetics. The physical sense of this model has been discussed by Hill and Xie (2011). In their work, the PEK parameters for sorption have been evaluated by the authors in terms of two Kelvin–Voigt elements arranged in series. Then, the force constant in the spring of each Kelvin–Voigt elements have been determined to be the equilibrium moisture content for each process, whereas the viscosity of the dashpot is represented by the time constant for each kinetic. Indeed, the adsorbed water vapor molecules exert a pressure within the cell wall leading to a dimensional change, which is equivalent to the extension of the spring in the Kelvin–Voigt model. The spring modulus therefore defines the water content of the system at infinite time (MC1, MC2). Moreover, the rate at which water molecules are adsorbed or desorbed by the system is a function of the viscosity of the dashpot in the model. The more rapidly the matrix is able to deform, the faster the rate of water ingress or egress into or out of the cell wall.

More recently Céline et al. (2013, 2014a,b) proposed to use Langmuir theory to explain the diffusion kinetics of several fibers in immersion. In this model developed by Carter and Kibler (1978) 35 years ago, the moisture absorption can be explained quantitatively by assuming that absorbed moisture consists of both mobile and bound phases. Molecules of the mobile phase

diffuse with a concentration and stress independent diffusion coefficient D_f , and are absorbed (become bound) with a probability per unit time γ at certain sites (for example: voids within the polymer, hydrogen bonding, and heterogeneous morphologies). Molecules are emitted from the bound phase, thereby becoming mobile, with a probability per unit time β . This model is well adapted with the structure and composition of plant fibers because it takes into account free and bound water.

Concerning the sorption isotherms, the water content is directly related to the relative humidity by following a sigmoidal relation, as described by Alix et al. (2009); Gouanvé et al. (2007). That kind of sorption isotherms are in a good agreement with the Park's model (Park, 1986). This model assumes the association of three mechanisms describing the three parts of the curve (Figure 6). It is often used to explain the sorption isotherms of hydrophilic and porous media, as cellulosic fibers (Bessadok et al., 2009). The first part of the curve could be related to Langmuir's mode ($RH < 10\%$). At these relative humidities, water is sorbed onto specific sites by hydrogen bonding. As previously discussed, the specific sites could be hydroxyl functions of amorphous cellulose and hemicelluloses or carboxylic function of pectin (Céline et al., 2014a). When relative humidity increases, there is a saturation of these specific sites of sorption. Then, the water concentration increases linearly with relative humidity as Henry's law describes (until $RH = 65\%$). This behavior could be explained by the porous structure of fibers where water is free to diffuse. The third part is well described by a power function that represents an aggregation phenomenon of water molecules. Indeed, at high relative humidity, water concentration is too important, and water molecules link together to form clusters. Moreover, it has been shown that fibers immersed in distilled water at room temperature could absorb 100–200% of water by weight depending on the kind of fiber (Symington et al., 2009; Céline et al., 2013) whereas in 80% relative humidity, the

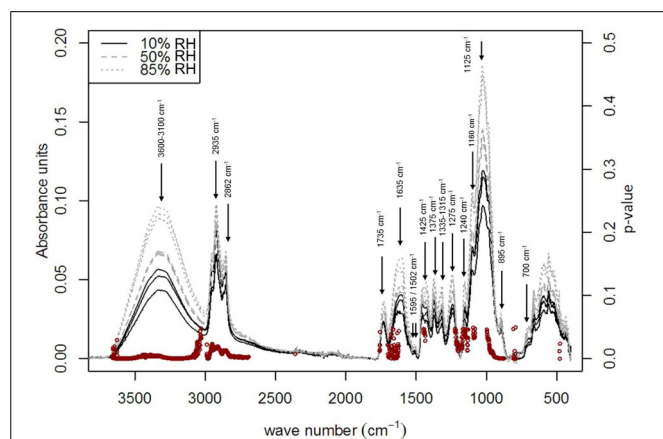


FIGURE 5 | Infrared spectra bands impacted by increasing relative humidity for sisal fiber. p -values scores (used with a threshold of 0.05), indicating significant impact of the water uptake on the FTIR bands, were marked using red dots (Céline et al., 2013, 2014a,b).

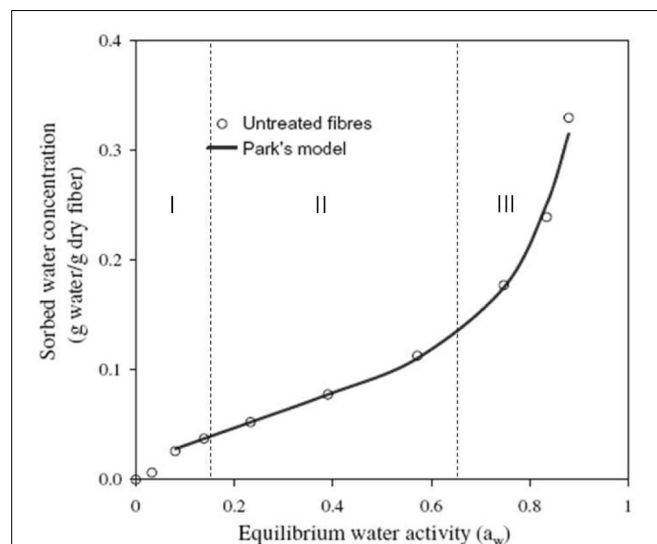


FIGURE 6 | Equilibrium water vapor sorption isotherm for modified agave fibers at 25°C [inspired by Bessadok et al. (2009)].

water content reaches about 10–15% (Watt and Kabir, 1975; Xie et al., 2011; Céline et al., 2013). Other sorption mechanisms could explain such a gap between immersion and vapor humidity conditions.

EFFECT OF WATER ON NATURAL FIBERS PROPERTIES

The moisture absorption in these hydrophilic fibers leads to a modification of their physical and chemical properties. Indeed, the interaction of water with hydrophilic materials may cause multiple phenomena as dimensional changes, modification of the mechanical, and chemical properties, and so on... Water can have a plasticization effect on the structure or, on the contrary, form stable hydrogen bonds leading to an anti-plasticizing effect (Hatakeyama and Hatakeyama, 1998).

DIMENSIONAL CHANGES

For synthetic composites, the relationship between the amount of water absorbed and the dimensional change is well documented (Weitsman, 2000). In the case of bio-composites, fibers are considered as anisotropic and hydrophilic, requiring to change models classically used for transversally isotropic and hydrophobic fibers. Quantitative information about the hydro-expansion coefficient of these fibers would be therefore an important factor for the development of new models adapted to these biomaterials. Some works have been published on the swelling of bio-composites (Madsen et al., 2012; Masoodi and Pillai, 2012). For instance, a study based on the deformation measurement of composites reinforced by hemp fibers, showed that the hydro-expansion coefficient increased with the fiber content in the material (Madsen et al., 2012). These results have also been observed by Masoodi and Pillai (2012) on a jute/epoxy bio-composite, showing that natural fibers have a strong influence on the dimensional changes of composites in which they are fitted. At the fiber scale, few study has been led to measure this coefficient despite the swelling is recognized to occur. Indeed, plant fibers have an unstable dimensional behavior. When subjected to a humid environment, they swell, resulting in the formation of internal stresses in the structure. For example, during their drying, natural fibers lose water so that shrinkage in their transverse direction could be observed. Moreover, dimensional changes of

natural fibers depend on their composition. Lee et al. (2010) studied the hygroscopic deformation of cellulose microfibrils by AFM (Atomic Force Microscopy). They showed that the characteristic times of water uptake and dimensional change of the sample are not in the same scale. Indeed, there is a delay time of swelling or shrinkage of cellulose fibrils after sorption phenomenon. Hygroscopic strains may be reversible -in this case they are predicted by the hydro-expansion coefficient- or irreversible due to structural defects.

Using a hygro-elastic model applied to an elementary fiber considered as a multilayer's hollow cylinder, (Neagu and Gamstedt, 2007) highlighted the parameters influencing the hydro-expansion coefficient on wood fibers. They found that the parameter the most influential is the microfibrillar angle of the S2 layer.

INFLUENCE ON MECHANICAL PROPERTIES

Concerning the influence of water on the mechanical properties, several authors showed a relationship between moisture and mechanical properties of plant fibers. Although this influence has been clearly demonstrated, the different results of the literature are not consistent altogether (Table 3). Indeed, Davies and Bruce (1998), observed experimentally a tendency to a decrease of the Young's modulus with increasing relative humidity for flax and nettle fibers (decrease of the Young modulus of flax fibers about 23% when relative humidity varies from 30 to 80%). This trend is also highlighted by Symington et al. (2009) for flax, and Ho and Ngo (2005) for hemp and coir fibers. However, other studies show an increase of the Young's modulus of fibers with relative humidity up to a specific threshold of water absorbed (Symington et al., 2009; Placet et al., 2012b). Particularly Placet et al. (2012b) show the young modulus of hemp fibers increases about 20% in the 25–80% relative humidity range. This increase in stiffness could be explained by a rearrangement of the microfibrils and the surrounding molecules acting as a matrix (Placet et al., 2012b). This rearrangement could be activated by the swelling of the fibers. Beyond a certain moisture content threshold, the decrease of the Young's modulus could be explained by the plasticization of the fiber. In fact, the formation of hydrogen bonds replacing bonds in hemicellulose macromolecular network could make the material

Table 3 | Literature review of the moisture absorption influence on the mechanical properties of plant fibers.

Kind of fiber	Hygroscopic conditions	Young's modulus evolution	Failure strength evolution	Elongation at break evolution	References
Flax and nettle	30, 40, 50, 60, and 70%	Decreases	Not significative effect		Davies and Bruce, 1998
Flax and sisal			Maximum for RH = 70%		Van Voorn et al., 2001
Flax	30, 66, 93%		Increases and stabilizes at RH = 66%		Stamboulis et al., 2001
Hemp	10, 25, 50, et 80%	increases	Maximum for 50 < HR < 70%		Placet et al., 2012b
Jute, flax, sisal, hemp, coir, agave	65, 90% et immersion	Increases until a threshold, then decreases (depend on the fiber)	Not significative effects	increases	Symington et al., 2009

more flexible and compliant. Astley and Donald (2001) studied this possible realignment of microfibrils during the hydration of flax fibers using X-ray diffraction. They highlighted a structural evolution of the fibers during dehydration. Thus, they proposed a model taking into account the reorganization of microfibrils during the water molecules desorption (microfibrillar angle varying from 15° to 11° for the dry sample).

Concerning the effect of water on the maximum tensile stress, the different results of the literature are consistent. It is often observed an increase in the stress at failure with the relative humidity, up to a threshold value of $RH = 50$ to 60% (Placet et al., 2012b) or $RH = 70\%$ (Van Voorn et al., 2001). Above these relative humidity, the tensile strength decreases. The absorption of water inside the fiber can lead to a rupture of the hydrogen bonds between the matrix of amorphous phase and the crystalline fraction of the fiber. This would reduce the tensile strength. The literature review reveals an increase of the fiber elongation with increasing the water content. Water acts as a plasticizer and softener of the structure.

Another phenomenon, highlighted by Mannan and Robbany (1996) and more recently by Placet et al. (2012b), is the rotation of the fibers in the presence of moisture. For a static loading, the authors showed that the rotation angle increased with relative humidity. In the same work Placet et al. (2012b) observed a remarkable increase in the stiffness of the fiber during tensile tests through relative humidity cycles of $RH = 50\%$ to $RH = 80\%$. The elastic modulus is increased by 250% from its initial value.

The diversity of these results in the literature is once again to be linked with the test conditions and variability factors of these fibers (growth conditions, extraction condition, storage condition...)

STRUCTURAL MODIFICATIONS

Structural modifications have been highlighted by several works. For example, in their research paper, Nakamura et al. (1983) suggest that the amorphous phase of the crystalline cellulose might become crystalline in the presence of bound water. Further tests by XRD show that the absorption of moisture in cellulose I results in an increase of the crystallinity degree. In connection with this increase of crystallinity, the authors showed an increase in tensile strength of hydrated cellulose I. The evolution of the crystalline structure of the fibers during drying was also investigated by Céline et al. (2014b) through calculating the total crystallinity index or TCI described by Nelson and O'Connor in the 60s (Nelson and O'Connor, 1964). This method supports the existence, in the cellulose infrared spectrum of both crystalline and amorphous characteristic bands. Then it is possible to estimate the fraction of the crystalline cellulose in the sample by determining the ratio of intensity of these bands. Results showed a decrease of the crystallinity degree with the decrease of the water content inside fibers, testifying the action of water on the cellulose macromolecular network. When water is removed from the sample, hydrogen bonds created between the water and the hydrophilic sites of the fibers are broken, leading to a relaxation of the macromolecular network and a decrease of the crystallinity degree. Recently, this hypothesis was confirmed by a XRD study on wood fibers submitted to hygroscopic cycles (Toba et al., 2013).

SUMMARY

Composites reinforced with natural fibers have developed significantly over the past years because of their biodegradability, low cost, low relative density, high specific mechanical properties, and renewable nature. These composites are predestined to find more and more applications in the near future since a lot of studies are led to understand and improve their properties. The understanding of the hygroscopic behavior of these materials is a key issue in order to use it in different weathering conditions. Many studies are examined, reviewed and highlighted in this paper regarding the link between the microstructure and the hydrophilic behavior of plant fibers, the influence of moisture on their properties as well as the final properties of the composites they reinforce. Water sorption in fibers and their composites has been found to significantly affect their dimensional and structural properties. Water sorbed in such fibers could be divided in two kinds of populations i.e., free and bound water. Free water is trapped inside the porous structure of plant fibers, whereas bound water could link to specific polar sites. These sites could be well identified by using spectroscopic techniques.

Further research is required to develop chemical or physical treatments which could reduce their water uptake. Moreover, investigations have to be conducted in order to take into account the swelling of fibers inside the composite and evaluate the internal stresses. In addition to that coupled diffusion model could be used in order to take into account the effects induced by mechanical states on the diffusion of moisture. Then, upcoming investigations could be focused on the use of more advanced multi-physics theoretical approaches dedicated to the modeling of the moisture uptake occurring while the heterogeneous, local swelling experienced by the reinforced polymer is accounted for Youssef et al. (2009, 2011); Sar et al. (2012, 2013).

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Electrochemical sensors and devices for heavy metals assay in water: the French groups' contribution

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A great challenge in the area of heavy metal trace detection is the development of electrochemical techniques and devices which are user-friendly, robust, selective, with low detection limits and allowing fast analyses. This review presents the major contribution of the French scientific academic community in the field of electrochemical sensors and electroanalytical methods within the last 20 years. From the well-known polarography to the up-to-date generation of functionalized interfaces, the different strategies dedicated to analytical performances improvement are exposed: stripping voltammetry, solid mercury-free electrode, ion selective sensor, carbon based materials, chemically modified electrodes, nano-structured surfaces. The paper particularly emphasizes their advantages and limits face to the last Water Frame Directive devoted to the Environmental Quality Standards for heavy metals. Recent trends on trace metal speciation as well as on automatic “on line” monitoring devices are also evoked.

Keywords: electrochemical detection, heavy metals, carbon electrode, polarography, mercury-free electrode, chemically modified electrode, ion selective electrode, speciation

INTRODUCTION

Like many other micropollutants such as drugs or cosmetics and their by-products, pesticides and industrial or household chemicals, heavy metals represent a growing environmental (Callender, 2004; Roy, 2010) and health (Musarrat et al., 2011; Prabhakar et al., 2012) problem. They may be considered as a major source of ecological issues due to their wide overspread in natural media (Mhatre, 1995). Although naturally produced throughout biogeochemical processes, heavy metals occurrence in the environment mainly originates from human activities: air emissions from coal-burning plants, smelters, waste incinerators, process wastes from mining, industrial and urban runoff all participate to their wide spreading (Friedman et al., 1993; Lindqvist, 1995; Nagajyoti et al., 2010). Once released to the environment, these

metals can remain for decades or centuries since they are not biodegradable. Depending on the contamination pathway, they appear at detectable levels in food resources such as vegetables, grains or fruits, and fish or shellfish throughout bioaccumulation all along the trophic chain, thus contaminating the final consumer—human being (Musarrat et al., 2011; Prabhakar et al., 2012). Another contamination way is direct intoxication from domestic environment, for instance lead traces in household plumbing and old house paints.

Once penetrated inside human organism by ingestion (drinking or eating), inhalation or skin contact, heavy metals may be responsible for nausea, vomiting, diarrhea or allergic reactions for short term or low-level exposure (Martin and Griswold, 2009). They can also cause severe diseases in the case of long term or chronic high-level exposure, such as reduced growth and development, cancers, organs or nervous system damages and even death (Prabhakar et al., 2012). There are over 50 elements that are classified as heavy metals, including transition metals, some metalloids, lanthanides and actinides. Among them 17 are considered to be both very toxic and relatively accessible. Lead (Pb), mercury (Hg), arsenic (As), and cadmium (Cd) are generally considered as leader elements in human poisoning even at trace level. The general population is mainly exposed to all these metals from air, drinking water and food, fish being a major source of mercury exposure. Moreover, smokers are highly exposed to cadmium (Järup, 2003). Some other heavy metals, including copper (Cu), zinc (Zn), nickel (Ni), cobalt (Co), selenium (Se), and bismuth (Bi) are known to play a vital role in physiological concentrations but can also be toxic in larger doses. Depending on the metal properties, the toxicity target may be different: kidneys

Abbreviations: ASV, anodic stripping voltammetry; BDD, boron doped diamond; BiFE, bismuth film electrode; CME, chemically modified electrode; CPE, carbon paste electrode; CSV, cathodic stripping voltammetry; CV, cyclic voltammetry; DAPV, differential alternative pulses voltammetry; DLC, diamond like carbon; DME, dropping mercury electrode; DPASV, differential pulse anodic stripping voltammetry; DPV, differential pulse voltammetry; FIA, flow injection analysis; GC, glassy carbon; HgFE, Hg film electrode; HMDE, hanging mercury drop electrode; ICP-MS, inductively coupled plasma mass spectroscopy; IEV, ion exchange voltammetry; ISE, ion selective electrode; LOD, limit of detection; LOQ, limit of quantification; LSASV, linear sweep anodic stripping voltammetry; LSSV, linear sweep stripping voltammetry; LSV, linear sweep voltammetry; MSWV, multiple square wave voltammetry; MWCNT, multi-walled carbon nanotube; NPV, normal pulse voltammetry; NPs, nanoparticles; SCP, stripping chronopotentiometry; SMCPE, silica-modified carbon paste electrode; SMDE, static mercury drop electrode; SPE, screen printed electrode; SSCP, stripping chronopotentiometry at scanned deposition potential; SWASV, square wave anodic stripping voltammetry; SWCNT, single-walled carbon nanotube; SWCSV, square wave cathodic stripping voltammetry; SWV, square wave voltammetry; TFME, thin film mercury electrodes; WFD, water frame directive.

Table 1 | Environmental Quality Standards for heavy metals (also called WFD).

Substance	CAS number	EQS-AA ^a	EQS-AA ^a	EQS MPC ^c	EQS MPC ^c
		Inside surface waters ^b nM [$\mu\text{g L}^{-1}$]	Other surface waters [$\mu\text{g L}^{-1}$]	Inside surface waters ^b nM [$\mu\text{g L}^{-1}$]	Other surface waters [$\mu\text{g L}^{-1}$]
Cadmium and its speciation (according to water hardness level ^d)	7440-43-9	≤ 0.71 (class 1)	1.78	≤ 4 (class 1)	≤ 4 (class 1)
		[0.08]	[0.2]	[0.45]	[0.45]
		0.71 (class 2)		4 (class 2)	4 (class 2)
		[0.08]		[0.45]	[0.45]
		0.8 (class 3)		5.34 (class 3)	5.34 (class 3)
		[0.09]		[0.6]	[0.6]
		1.33 (class 4)		8.09 (class 4)	8.09 (class 4)
Lead and its speciation	7439-92-1	[0.15]		[0.9]	[0.9]
		2.22 (class 5)		13.3 (class 5)	13.3 (class 5)
		[0.25]		[1.5]	[1.5]
Mercury and its speciation	7439-97-6	34.7	34.7	Groundless	Groundless
		[7.2]	[7.2]		
Nickel and its speciation	7440-02-0	0.25	0.25	0.35	0.35
		[0.05]	[0.05]	[0.05]	[0.05]
		341	341	Groundless	Groundless
		[20]	[20]		

^aEnvironmental Quality Standard—annual average.

^bInside surface waters include rivers, lakes and also water masses (artificial or seriously modified) related to them.

^cEnvironmental Quality Standard—maximal permissible concentration.

^dFor cadmium and its compounds, EQS—AA values are functions of water hardness according to the five classes as follows: class 1: $<40 \text{ mg CaCO}_3 \text{ L}^{-1}$; class 2: $40\text{--}50 \text{ mg CaCO}_3 \text{ L}^{-1}$; class 3: $50\text{--}100 \text{ mg CaCO}_3 \text{ L}^{-1}$; class 4: $100\text{--}200 \text{ mg CaCO}_3 \text{ L}^{-1}$; class 5: $\geq 200 \text{ mg CaCO}_3 \text{ L}^{-1}$.

^eMolar concentrations have been chosen as reference unit for the sake of comparison facility and regarding to the standards of the WFD (Water Frame Directive), even if this latter uses mass concentrations.

or liver are damaged by Cu, Hg, and Pb ions while these latter two also attack central nervous system (Vallee and Ulmer, 1972; Hamilton et al., 1998). Thus, toxicity levels are related to the nature of the metal but also to its biological and biogeochemical roles, both being strongly dependent on its speciation, i.e., the different available physico-chemical forms, namely particulate (size $> 1 \mu\text{m}$), colloidal ($1 \text{ nm}\text{--}1 \mu\text{m}$) and dissolved ($\leq 1 \text{ nm}$) species. These latter include free metal ions, simple inorganic complexes and complexes bearing anthropogenic and natural organic ligands (Templeton et al., 2000). Hence speciation information on heavy metals of concern appears to be data of particular relevance (Kot and Namiesnik, 2000).

In 2000, a new European directive (“Water Frame Directive,” WFD, see Table 1) (European Directive 2000/60/EC) particularly pointed out four heavy metals (Hg, Cd, Pb, Ni) and has established their maximal authorized as well as annual average concentration values in surface waters. As a consequence, environmental monitoring of heavy metals is of critical importance for both ecological assessments and public health preservation. In answer there is an urgent need for *in situ*, real-time, and highly-sensitive sensors in order to multiply control points dedicated to early warning pollution alert (Suib, 2013).

Heavy metals trace detection is mainly performed using spectroscopic techniques: atomic absorption spectroscopy (Kenawy et al., 2000; Pohl, 2009), inductively coupled plasma mass

spectroscopy (ICP-MS) (Caroli et al., 1999; Silva et al., 2009), X-ray fluorescence and neutron activation analysis are the most commonly used. Their main advantages are their versatility since they are suitable for a large panel of elements, their sensitivity and their limit of detection (LOD) in the femtomolar range. However they suffer from several major drawbacks: expensive materials are required and qualified operators are needed to perform the multi-step sample preparation and complex analytical procedures, which are unsuitable for on-site and on time measurements necessary to prevent transient phenomena monitoring. Finally, only total metal concentration can be determined, and speciation data can be reached only by associating supplementary extraction and separation techniques such as chromatography to the spectroscopic detection (Feldmann et al., 2009). These additional steps significantly increase the risk of contamination of the sample and some modifications of the speciation may occur during sample storage or handling.

On the contrary electrochemistry represents an interesting alternative due to its numerous advantages. Electrochemical devices are mostly user-friendly since they require simple procedures. They are also reagentless, low cost, and well-suited for miniaturization and automatic *in situ* measurements with minimal sample changes. Thus, contamination by reagents or losses by adsorption on containers are drastically decreased. Electrochemical systems also allow quite fast analyses with

experimental data obtained mostly in real time or in a few minutes. Hence, on-line monitoring of water samples becomes possible, providing dynamic data of relevance for biogeochemical survey. Nevertheless specific developments are still required for such applications, particularly to improve sensitivity, LODs and automation. In this way, a large number of electrochemical techniques with different imposed potential or current modulations have been developed such as differential pulse voltammetry (DPV), square wave voltammetry (SWV) or stripping chronopotentiometry (SCP). Electrochemical sensors also allow high temporal resolution measurements to be obtained when associated to flow injection analysis (FIA) or flow electrochemical analysis cells, thus providing continuous *in situ* measurements. Another analytical performance of high relevance with respect to heavy metals detection concerns the selectivity. In complex media, the signal of the analytical target often experiences interferences due to the presence of other species (sometimes other heavy metals). To solve this problem, several surface functionalization strategies have been developed for many years to improve sensors selectivity.

Many pioneering researchers coming from several countries have initiated and intensified works dealing with electrochemical techniques for heavy metals detection and assay in natural media: Jaroslav Heyrovsky (Czechoslovakia) (Heyrovsky, 1960), Joseph Wang (USA) (Wang et al., 1995), Richard G. Compton (UK) (Agra-Gutierrez and Compton, 1998), George Luther III (USA) (Luther III and Ferdelman, 1993), Laura Sigg (Switzerland) (Goncalves et al., 1985), Arben Merkoçi (Spain) (Aragay and Merkoçi, 2012), Marco Mascini (Italy) (Voccia et al., 2012), and so on. French contribution to this topic is also noticeable. This is mainly due to the voluntary policy lead since 1998 and the Aarhus protocol in which France contracted to limit its release of Pb, Cd, and Hg at a lower level than that recorded in 1990 (Commissariat général au développement durable, 2012). This goal was reached before the protocol came into effect in 2003, but the situation is still worrying: over the 2007–2009 period, 25 heavy metals have been detected in more than 10% of the analyses performed in French rivers (Commissariat général au développement durable, 2011), whereas the contamination of mussels and oysters, which constitute a good indicator of coastal water pollution, remained stable over the last 3 decades (Commissariat général au développement durable, 2012). This review provides a survey of French groups' contribution to the development of electrochemical sensors and methods aiming at heavy metals detection. The paper particularly emphasizes the multidisciplinary of French scientific investigations through the description of the electrochemical techniques and the evaluation of the corresponding analytical performances.

POLAROGRAPHY

Classical technique

Polarography has been certainly the most studied and commonly used electrochemical technique throughout the 20th century since the pioneering work of Heyrovsky in 1922 (Heyrovsky, 1922). This is undoubtedly the consequence of the particular properties of the mercury electrode: continuous renewal of the active surface area, wide cathodic potential window due to the

high overpotential corresponding to hydrogen evolution, control of the hydrodynamic conditions by means of mercury drop. These characteristics make polarography a very powerful electrochemical technique for the study of inorganic, organic, organometallic, or biological compounds, not only from a theoretical point of view but also for analytical applications. In this frame the assay of heavy metals has been the subject of numerous papers due to the large inclination of mercury to form amalgams with major metal compounds. For concentrations higher than 10^{-5} M linear sweep voltammetry (LSV) on a dropping mercury electrode (DME) or on a static mercury drop electrode (SMDE) generated at the end of a glass capillary is well-suited. For lower concentrations, the faradic current becomes smaller and the double-layer charging current is not negligible anymore. Pulse techniques, i.e., normal pulse (NPV), differential pulse (DPV) and square wave (SWV) voltammetries have been favored to partially suppress the background current and thus improve the LOD. In the case of trace metals detection these potential pulse programs have been associated with anodic (ASV) or cathodic (CSV) stripping voltammetries on a hanging mercury drop electrode (HMDE) inside which the analyte is pre-concentrated by constant potential electrolysis prior to analysis. The resulting methods, i.e., LSASV, DPASV and SWASV and their combination, allow LODs down to 10^{-12} M to be reached (Bard and Faulkner, 2001). For instance Superville et al. assembled an automatic anodic stripping analysis system with a SMDE to undertake a real-time routine analysis of the dynamic behavior of trace metals (Zn, Pb, Cd) in river, pond and seawater (Superville et al., 2011) (see **Table 2** for quantitative features). Furthermore a CSV was included to estimate simultaneously the concentration of dissolved oxygen and reduced sulfur species. Magnier et al. perfected a procedure to assay lead and zinc by ASV and copper by CSV in certified reference freshwater and in the French Deûle river, Cu analysis requiring the complexation with 8-hydroxyquinoline (Magnier et al., 2011).

The need for determination of very low concentrations has favored the development of specific electrochemical techniques with new potential perturbation modes providing high resolution and/or improved sensitivity. In this way Zlatev et al. particularly emphasized the advantages of differential alternative pulses voltammetry (DAPV) on HMDE to analyse mixtures of species exhibiting very close half-wave potentials (like Pb^{2+} and Tl^{+} or Co^{2+} and Ni^{2+}) or species couples with high concentration ratios (Zlatev et al., 2006) for which the analysis by DPV is hampered by complete peaks overlapping. DAPV takes advantage of the high resolution power of the second-order voltammetric techniques (as radio-frequency polarography) combined with the high sensitivity and instrumental simplicity of DPV or SWV. DAPV principle is based on the superimposition on the main electrode potential E of a pair of single successive rectangular pulses characterized by small, equal amplitudes ($<RT/nF$) and durations (from 1 to 100 ms) but opposite polarities. The overall current recorded at this potential E corresponds to the deviation of the average of the corresponding cathodic and anodic amperometric responses. Thus the resulting current-potential curve exhibits the typical shape of a first-order peak derivative passing three times through zero (for potential ranges corresponding to residual and diffusion-limited currents and for the electrode

Table 2 | Summary of analytical performances and experimental conditions obtained for heavy metals detection in French scientific academic community.

Classification	References	Electrochemical platform	Detection medium	Technique	Analyte(s)	LOD	Linear concentration range	Analyte pretreatment conditions
POLAROGRAPHY								
	Magnier et al., 2011	HMDE	Online monitoring river	DPASV	Total Zn	2.91 nM	12.4–23.2 nM	30 s at –1.3V
					Total Pb	0.03 nM	1.7–3.2 nM	60 s at –0.7V
				DPCSV	Total Cu	0.6 nM	4.9–7.6 nM	30 s at –1.1 V followed by an adsorption step at –0.25V during 15 s
	Riso et al., 2006	HgFE	Water samples (treated)	SCP	Fe(III)	1.5 nM	NC	6 cycles of: 0.04 V (9 s) and –0.4V (1 s)
	Tanguy et al., 2010	HgFE	Seawater samples (treated)	SCP	Sb(III)	70 pM	depending on sample	300 s at –0.45 V
	Sladkov et al., 2003	HgFE	0.1 M HNO ₃	SWCSV	Se(IV)	0.8 nM	1–1000 nM	300 s at –0.45 V
					Cu	0.7 nM		
					Pb	14 pM	NC	15 min at –1.1 V
	Riso et al., 1997	HgFE	Seawater	SCP	Cd	9 pM		
	Cugnet et al., 2009	SPμEAs	0.2 M acetate (pH = 4.5)	SWASV	Cd(II)	11.6 nM	11.6–89 nM	300 s at –1 V
	Parat et al., 2011a	HMDE	0.1 M KNO ₃	AGNES-SCP	Zn ²⁺	4 nM		
					Cd ²⁺	2.9 nM	at least 25–100 nM	1400 s total with complex procedure
					Pb ²⁺	4.1 nM		
	Parat et al., 2007	Membrane and Hg film SPE	0.2 M acetate (pH = 4–7)	SWASV	Cd(II)	2 nM	5–100 nM	60 s at –1 V
	Munteanu et al., 2009	Mercury monolayer carbon fiber electrode	NC	SWASV	Pb(II)	80 fM	1–10 pM	1 s at –1.2V
	Parat et al., 2011b	Hg film SPE	0.2 M acetate (pH = 4.6)	SSCP	Cd	2.2 nM	NC	60 s at –1 V
	Zaouak et al., 2010a	Hg film SPE	0.2 M acetate (pH = 4.5)	SWASV	Cd	1.78 nM	1.78–356 nM	60 s at –1 V
BI FILMS								
	Guo et al., 2005	GC/BiFE	Milk vetch in 0.2 M KSCN	ASV	Zn(II)	9.6 nM	500–3000 nM	120 s at –1.4 V

(Continued)

Table 2 | Continued

Classification	References	Electrochemical platform	Detection medium	Technique	Analyte(s)	LOD	Linear concentration range	Analyte pretreatment conditions
	Legéai et al., 2005	GC/BiFE	0.125 M HNO ₃ + 0.04 M H ₂ NSO ₃ H	DPASV	Cd(II)	~10 nM	20–1000 nM	1200 s at –0.95 V
	Legéai et al., 2006	Cu/Bi film electrode	0.01 M ammonia buffer (pH = 9)	SWASV	Ni ²⁺	NC	10–1000 nM	900 s at –0.7 V
	Legéai and Vitori, 2006	Cu/Nafion/Bi electrode	0.01 M NaCl + 0.001 M NaHCO ₃	DPASV	Cd ²⁺ Pb ²⁺	6.05 nM 3 nM	178–107 nM 9.65–86.9 nM	300 s at –0.95 V
	Urbanova et al., 2010	Highly porous Bi film electrodes	0.1 M acetate buffer (pH = 4.5)	DPASV	Cd(II) Pb(II)	5.34 nM 6.27 nM	178–1160 nM 96.5–627 nM	90 s at –0.95 V
	Zaouak et al., 2009	Bi-Coated SP _μ E	0.2 M acetate buffer (pH = 4.5)	SWASV	Cd(II)	11.6 nM	45–400 nM	120 s at –1 V
	Lu et al., 2010	Bi doped carbon SPE	Air	SWASV	Pb(II) as vapor	1 ng	10–80 ng	120 s at –1.2 V
CARBON ELECTRODE MATERIALS								
DLC/GC								
	Feier et al., 2012	Graphite felt	0.1 M NaBF ₄	LSASV	Zn(II)	50 nM	1–100 μM	300 s at –1.4 V
	Nasraoui et al., 2009	Graphite felt	0.1 M LiClO ₄	LSASV	Pb(II)	1 nM	10–500 nM	300 s at –1 V
	Khadro et al., 2009	GC electrode	0.1 M HCl 0.1 M acetic buffer	DPASV	Ni(II) Hg(II)	2.56 nM 0.15 nM	8.52–9370 nM 0.5–1740 nM	60 s at –1 V
	Khadro et al., 2011	B-doped DLC	Acetate (pH = 4.2)	SWASV	Cd(II) Pb(II) Ni(II) Hg(II)	4.83 nM 8.9 nM 34.1 nM 4.99 nM	4.83–121 nM 8.9–222 nM 34.1–256 nM 5–125 nM	90 s at –1.3 V
BDD								
	Le et al., 2012	BDD	acetate pH = 5.2	SWASV	Pb(II)	19.3 nM	96.5–480 nM	600 s at –1 V
	El Tall et al., 2007	BDD	0.01 M acetate	DPASV	Cu(II) Pb(II) Zn(II) Cd(II)	14.2 nM 5.55 nM 25.5 nM 3.2 nM	47–315 nM 18–217 nM 77–305 nM 11–222 nM	60 s at –1.9 V

(Continued)

Table 2 | Continued

Classification	References	Electrochemical platform	Detection medium	Technique	Analyte(s)	LOD	Linear concentration range	Analyte pretreatment conditions
	Sbartai et al., 2012	BDD	Potassium citrate / HCl	DPASV	Cd(II)	3.29 nM	NF	20 s at −1.7 V
					Pb(II)	26.5 nM		
					Ni(II)	116 nM		
					Hg(II)	11.5 nM		
ISEs								
CALIXARENE								
	Yaftian et al., 2006	Calix[4]arene	Complex (pH = 3.5–5)	SCP	Pb(II)	1.4 μM	10 μM–10 mM	No accumulation and electrolysis
	Yaftian et al., 2007	Calix[4]arene	Complex (pH = 3–7)	SCP	Pb(II)	4 nM	10 nM–100 μM	No accumulation and electrolysis
CHALCOGENIDE								
	Cali et al., 2002	Cu-As-S	KNO ₃	SCP	Cu(II)	1 μM	2 μM–10 mM	No accumulation and electrolysis
	Essi and Pradel, 2011a,b	Cu-Ag-S	Complex (pH = 3–5)	SCP	Cu(II)	1 μM	NC	No accumulation and electrolysis
	Mear et al., 2005	Ge ₂₈ Se ₆₀ Sb ₁₂	KNO ₃ (pH = 3)	SCP	Cd(II)	1 μM	1 μM–10 mM	No accumulation and electrolysis
CMEs								
MINERALS								
	Walcarius et al., 1999a	Silica modified CPE	0.2 M HNO ₃	SWASV	Cu(II)	2 nM	5 nM–5 μM	600 s accumulation followed by 30 s at −0.5 V
	Walcarius et al., 2000	Silica-modified electrode	0.1 M HNO ₃	SWASV	Hg(II)	50 nM	200 nM–10 μM	600 s accumulation at open circuit followed by 60 s at −0.5 V
	Walcarius et al., 1999b	Several silica/hybrid CPE with amine functionalization	0.05 M acetic acid + 0.05 M NaNO ₃	LSASV	Cu(II)	NC	unclear	Several accumulation time and 240 s at −0.4 V
	Etienne et al., 2001	Organically modified silica	0.1 M sodium acetate	SWASV	Cu(II)	3 nM	50–200 nM	60 s at −0.5 V
	Sayen et al., 2003	Carnosine silica hybrid material modified CPE	0.1 M NaNO ₃ + 0.01 M HNO ₃	DPASV	Cu(II)	4 nM	50–1000 nM	90 s at −0.5 V
	Walcarius and Sibottier, 2005	Amine-functionalized porous silica films on Au	0.1 M HNO ₃ + 0.1 M NaNO ₃ in 95% ethanol	DPASV	Cu(II)	40 nM	0.1–10 μM	600 s accumulation followed by 60 s at −0.4 V
	Etienne et al., 2007	Surfactant-templated thiol-functionalized silica thin films	0.5 M HCl	DPASV	Ag(I)	6 nM	0.2–10 μM	960 s accumulation followed by 60 s at −0.6 V
(Continued)								

(Continued)

Table 2 | Continued

Classification	References	Electrochemical platform	Detection medium	Technique	Analyte(s)	LOD	Linear concentration range	Analyte pretreatment conditions
	Sanchez and Walcarius, 2010	GC/MTTZ	0.1 M HCl	SWASV	Hg(II)	2 nM	50 nM–1 μ M	300 s at –0.4 V
	Walcarus et al., 1998	Mesoporous pure silica modified carbon paste electrode	0.2 M HNO ₃	SWASV (or CV for larger amounts)	Cu(II) Hg(II)	30 nM 50 nM	NC NC	300 s accumulation followed by 60 s at –0.5 V
	Tonle et al., 2005	Clays grafted with organic chelating groups (thiol or amine) modified CPE	0.1 M HNO ₃	DPASV	Hg(II)	68 nM (thiol) 87 nM (amine)	100–700 nM	180 s accumulation followed by 60 s at –0.4 V (or –0.6 V depending on the medium)
	Tonle et al., 2011	Thiol-functionalized clay modified CPE	0.2 M HNO ₃	SWASV	Pb(II)	60 nM	0.3–10 μ M	600 s accumulation followed by 60 s at –0.9 V
	Tchinda et al., 2007	GC/PCH-SH	0.1 M HCl + 5% thiourea	DPASV	Hg(II)	0.4 nM	4–20 nM and 50–80 nM	1200 s accumulation followed by 180 s at –0.7 V
MACROCYCLIC COMPOUNDS								
	Rouis et al., 2013	β -ketimine calix[4]arene on ITO	0.05 M ammonium acetate (pH = 7)	Impedance	Hg ²⁺	NC	0.1 nM–0.5 μ M	
	Goubert-Renaudin et al., 2009a	Cyclam-functionalized silica CPE	3 M HNO ₃	SWASV	Cu(II)	0.8 nM	2–100 μ M	1800 s accumulation followed by 60 s at –0.5 V
	Goubert-Renaudin et al., 2009b	(TETAM) grafted to silica gel and ordered mesoporous silica	0.1 M ammonium acetate buffer (pH = 7)	SWASV	Pb(II)	2.7 nM	10–100 nM	900 s accumulation followed by 60 s at –0.8 V
	Nasraoui et al., 2010a	TETAM-modified graphite felt electrode	0.1 M aqueous solution of LiClO ₄	LSASV	Pb(II)	25 nM	100–250 nM	around 1800 s accumulation followed by 300 s at –1 V
	Nasraoui et al., 2010b	Cyclam-modified graphite felt	0.5 M H ₂ SO ₄	LSASV	Pb(II)	25 nM		several accumulation time followed by 300 s at –1 V
	Parat et al., 2006	Hg film modified SPE	0.1 M KNO ₃	LSASV	Cd(II) Pb(II)	6 nM 8 nM	NC	120 s at –1 V

(Continued)

Table 2 | Continued

Classification	References	Electrochemical platform	Detection medium	Technique	Analyte(s)	LOD	Linear concentration range	Analyte pretreatment conditions
POLYMERS								
	Betelu et al., 2007	Hg film + membrane modified SPE	0.01 M NaHCO ₃	LSASV	Cd(II) Pb(II)	NC	NC	120 s at -1 V
	Heitzmann et al., 2007	Poly(pyrrole-EDTA like) film	0.1 M buffer (pH = 5)	SWASV	Cd(II), Pb(II) and Cu(II)	NC	NC	600 s accumulation followed by 40 s at -1.2 V for Pb(II) and -0.9 V for Cu(II)
	Buica et al., 2009a	Poly(EDTA-like) Film	0.1 M acetate buffer (pH = 4.5)	DPASV	Hg(II) Cu(II)	0.5 nM	NC	600 s accumulation followed by 60 s at -0.4 V
	Buica et al., 2009b	Poly(pyrrole-EDTA) modified electrode	0.1 M acetate buffer (pH = 4.5)	DPASV	Hg(II)	10 nM (imprinted polymers)	10–1000 nM (imprinted polymers)	600 s accumulation followed by 180 s at -1.8 V
	Heitzmann et al., 2005	Poly(pyrrole-malonic acid) film modified carbon electrode	0.2 M acetate buffer (pH = 4.4)	SWASV	Pb(II)	0.5 nM	10–1000 nM	600 s accumulation followed by 40 s at -0.9 V
					Cu(II)	5 nM	25–250 nM	
					Hg(II)	50 nM	NC	
					Cd(II)	0.2 µM	1–10 µM	600 s accumulation followed by 40 s at -1.1 V
	Pereira et al., 2011	Complexing polymer films	0.1 M acetate buffer (pH = 4.4)	SWASV	Pb(II)	0.5 nM	10–1000 nM	600 s accumulation followed by 40 s at -0.9 V or -1.1 V for Cd(II)
					Cu(II)	5 nM	25–250 nM	
					Hg(II)	100 nM	100–1000 nM	
					Cd(II)	500 nM	100–10000 nM	
	Rivas et al., 2006	Complexing polymer films	0.1 M acetate buffer (pH = 4.8)	SWASV	Pb(II)	NC	0.01–5 mM	600 s accumulation followed by 40 s at -0.6 V
	Bessbousse et al., 2011	Nanoporous β-PVDF membrane electrode	0.1 M sodium acetate	SWASV	Pb(II)	0.63 nM	NC	30 min equilibrium followed by 100 s at -0.8 V
	Zejji et al., 2007	Polythiophene film	0.2 M KNO ₃ (pH = 5)	DPASV	Ag(I)	0.56 µM	0.65–9.3 µM	120 s at -0.5 V
	Yasri et al., 2011	GC/PEDOT:PSS	HCl (pH = 2.2)	CA	Pb(II)	0.19 nM	2–100 nM	30 s at -0.65 V

(Continued)

Table 2 | Continued

Classification	References	Electrochemical platform	Detection medium	Technique	Analyte(s)	LOD	Linear concentration range	Analyte pretreatment conditions
NPs								
	Ottakam Thotiyil et al., 2012	Au/MPS-(PDDA-AuNPs)	Phosphate buffer (pH = 8)	DPASV	As(III)	0.48 μ M	NC	
	Hezard et al., 2012a	GC + AuNPs	0.01 M HCl	SWASV	Hg(II)	0.42 nM	0.64–4 nM	300 s at 0 V
	Hezard et al., 2012b	GC + AuNPs	0.01 M HCl	SWASV	Hg(II)	0.4 nM	0.8–9.9 nM	300 s at 0 V
BIOSENSORS								
	Chouteau et al., 2004	Alkaline phosphatase	10 mM Tris-HCl buffer (pH = 8.5) / 1 mM MgCl ₂	Conductometry	Cd ²⁺	8.9 nM	NC	
	Chouteau et al., 2005	Alkaline phosphatase Acetylcholinesterase	10 mM Tris-HCl buffer (pH = 8.5) / 1 mM MgCl ₂	Conductometry	Cd ²⁺ Zn ²⁺	89 nM 0.15 μ M	NC	30 min incubation
	Tekaya et al., 2013	Alkaline phosphatase	5 mM HEPES buffer (pH = 8.1)	Conductometry	Cd ²⁺	10 ^{–20} M	NC	24 h incubation
	Soldatkin et al., 2012	Invertase, mutarotase, glucose oxidase	5 mM phosphate buffer (pH = 6.5)	Conductometry	Hg ²⁺ Hg ²⁺	25 nM	NC	20 min incubation
	Mohammadi et al., 2005	Invertase, mutarotase, glucose oxidase	0.1 M phosphate buffer (pH = 6)	Amperometry	Ag ⁺ Hg(II)	100 nM NC	10 nM–1 μ M	20 min incubation at pH = 4
	Gayet et al., 1993	Lactate dehydrogenase Lactate oxidase	0.1 M Tris buffer (pH = 9)	Amperometry	Hg(II) Ag ⁺ Cd ²⁺ Zn ²⁺ Pb ²⁺ Cu ²⁺	1 μ M 0.1 μ M 10 μ M 10 μ M 50 μ M 250 μ M	NC	5 min incubation

* All parameters given are the ones for the best LODs.

potential equal to the half-wave potential) with peak amplitudes proportional to the electroactive species concentration. The resolution power of DAPV was highlighted through the analysis of a solution containing Pb^{2+} , Tl^+ , In^{3+} , and Cd^{2+} . The sensitivity and the LOD (54 nM) were found to be similar to those obtained using classical DPV but with species having half-wave potentials difference in the range from 28 to 50 mV and concentration ratios from 1:1 up to 80:1 without any preliminary preparation of the sample.

Mercury film

Despite very good analytical performances in terms of sensitivity and stability of the response vs. time, the low vapor pressure and the high toxicity of mercury encouraged extensive researches on polarographic methods involving reduced amounts of mercury. One way consists in thin film mercury electrodes (TFME) electrodeposited on solid state materials like glassy carbon (GC). The group of Riso used SCP with low constant current for the quantification of Fe(III) in estuarine and coastal filtered waters (Riso et al., 2006). The procedure was proved to be highly sensitive but analysis required several pre-treatment steps, i.e., filtration of the sample and complexation with solochrome violet. They also succeeded in detecting ultra-trace (70 pM) Sb(III) in seawater by using the same electrochemical method. The application of a double electrolysis potential during the pre-concentration step allowed the analysis to be independent from the Cu level (Tanguy et al., 2010).

For the determination of Se(IV), insufficient reproducibility and sensitivity of Hg film was observed by Sladkov et al. (2003). This problem was overcome by incorporating Cu(II) ions during the plating procedure on GC electrode surface. The metallic Cu dissolved in the Hg film was found to play an important role in peak current enhancement. A LOD 0.8 nM was reached by SWCSV and the relative standard deviation was 5.2% ($n = 5$) for 1 μM Se(IV).

A potentiometric stripping method has been proposed by Riso et al. for the simultaneous measurement of Cu, Pb, and Cd in ocean waters (Riso et al., 1997). The mercury coating was electrodeposited *in situ* on a GC rotating electrode at the beginning of each analysis by applying a potential step at -1.1 V/SCE for 10 min. Then an electrolysis-stripping cycle was carried out. Metals concentrations were compared with a reference standard solution containing all three metals. The obtained LODs were 0.7 nM, 15 pM and 9 pM for Cu, Pb, and Cd, respectively. However, it has to be noticed that the total duration of the analysis was quite long, about 75 min.

In order to approach solid mercury-free electrodes, Munteanu et al. worked on the electrodeposition of a mercury monolayer by constant potential electrolysis with increasing electrolysis time (Munteanu et al., 2009). An exceptional sensitivity for Pb^{2+} assay was obtained when the mercury monolayer-on-carbon electrode was used with fast ($v > 1$ kV/s) ASV. This result was revealed to be due to the ionization of Pb atoms in the mercury layer, which catalyzes the oxidation of atomic hydrogen adsorbed on the Hg layer. A remarkable LOD of 80 fM was recorded on a cylinder electrode with a 1 s preconcentration time.

Mercury screen printed electrode (HgSPE)

Potin-Gautier's group has developed an alternative strategy based on mercury micro arrays screen printed electrodes (SPE). This micro system allowed mass transport of the analytes to be enhanced compared to macro systems. This device was successfully tested for Cd^{2+} detection in synthetic and river samples, providing a LOD of 11.6 nM using SWASV (Cugnet et al., 2009). More recently SCP was implemented as the second stage of the electrochemical technique "Absence of Gradients and Nernstian Equilibrium Stripping" (AGNES-SCP) for the determination of free metal concentration, namely Zn^{2+} , Cd^{2+} , and Pb^{2+} , with both an HMDE and a SPE (Parat et al., 2011a). The results showed higher sensitivities and lower LOD and LOQ (in the nanomolar range) with SPE, which was linked to a higher product of mercury volume times the gain (AGNES parameter). Finally, SPE was modified by a microwell for the assay of labile Cd^{2+} , thus reducing the sample volume down to 200 μL . A LOD of 2 nM was reached using SWASV with only a 60 s preconcentration step. Unfortunately, the performances of this electrode were pH-dependent out of the pH range 4–7 (Parat et al., 2007). Nevertheless all these mercury-based techniques are promised to disappear in a few years since no mercury will be authorized from 2015 (European Directive 2008/105/EC).

BISMUTH

Bismuth film electrode (BiFE) is often considered to be a good alternative to Hg electrode and has been extensively used for electroanalysis. More "environmentally friendly" and less toxic than Hg, Bi is considered to be a safe material, as it is a non-carcinogenic element (except for foetus and embryo) (Svancara et al., 2010). However, in high doses, it presents similar toxicity to other heavy metals apart from these effects are much more reversible. The main advantage of Bi with respect to trace analysis is its capability to form binary or multi-component alloys with numerous other heavy metals (Svancara et al., 2010). It has also the particularity to be the most diamagnetic metal, thus avoiding conductance problems. Another interest of Bi compared to Hg is its insensitivity to dissolved oxygen, thus making unnecessary any deaeration step. Most of the time, the Bi film is plated before analysis onto the electrode by potentiostatic reduction of a Bi(III) solution (Wang et al., 2001), although codeposition during trace metal reduction has been also reported (Wang et al., 2000). BiFE performances compare favorably with Hg electrodes, affording high sensitivity and well-defined stripping signals. For instance, Guo et al. compared the anodic stripping voltammetric response of HgFE and BiFE obtained in a 10 mM Zn(II) solution (Guo et al., 2005). The BiFE presented a well-defined and higher stripping peak compared to HgFE. Its sensitivity was found to be twice higher than that obtained on HgFE. This electrode was then used to detect zinc contained in milk vetch used in traditional Chinese medicine. The response was linear in the range from 0.5 to 3 μM and a LOD of 10 nM was reported. Nevertheless, it has to be noticed that the cathodic limit of the potential window is higher than on Hg.

In 2005, Legeai et al. proposed an interesting alternative to the classical Pt or GC substrates by electrodepositing Bi film onto Cu since the adherence of the film was found to be better in this

latter case (Legeai et al., 2005). By using DPASV as the detection method, this BiFE exhibited very good performances toward Cd^{2+} assay in acidified tap water with a linear concentration range between 10^{-8} and 10^{-6} M. It was also successfully tested for the simultaneous determination of Cd^{2+} , Zn^{2+} and Pb^{2+} ions at 10^{-5} M. Good accuracy ($<5\%$) was confirmed by comparison of the electrochemical data with those obtained by ICP-MS and mercury-drop electrode on aquatic plant extracts. These results were later generalized to Ni^{2+} analysis, by using adsorptive stripping voltammetry and dimethylglyoxime as the complexing agent (Legeai et al., 2006). However, BiFEs suffer a major drawback since the formation of Bi hydroxides, which occurs at pH higher than 4.3, makes the analytical results irreproducible due to film surface changes. To overcome this problem, Legeai et al. used a Nafion-coated Cu substrate for Bi electrodeposition and observed that the Bi particles which were incorporated into the membrane did not experience any hydroxylation reaction (Legeai and Vittori, 2006). This system was successfully used for Cd^{2+} and Pb^{2+} detection and exhibited good stability (only 10% decrease after 23 days).

In a very close approach, Urbanova et al. succeeded in increasing the active surface area of the electrode by depositing Bi onto a polystyrene spheres template (Urbanova et al., 2010). After polystyrene dissolution by toluene washing, the resulting ordered porous film exhibited an original three-dimensional structure which significantly enhanced the analytical performances. These latter were found to be strongly correlated to the Bi film thickness. By using the thickest porous film (2.2 μm thickness) and DPASV method, linear concentration ranges between 178 nM and 1.16 μM for Cd^{2+} ions and between 96.5 and 627 nM for Pb^{2+} ions were obtained (Figure 1). The LODs were 5.3 nM and 6.3 nM respectively, with a pre-concentration time of 300 s. The standard deviations were lower than 4% in both cases.

Zaouak et al. reported comparable performances by using a Bi film electrodeposited on carbon screen-printed microband

electrode and SWASV (linear range from 10 to 400 nM and LOD 5.34 nM with 8% reproducibility and 3% repeatability in synthetic solution) (Zaouak et al., 2009). This system was successfully tested on non-treated river water sample but exhibited poor stability in this case (4% decrease in 10 min) due to both Bi film hydroxylation and biofouling. To overcome this drawback, the authors suggested the renewing of the microband after each measurement.

Finally, the best performances of Bi electrodes were reported by Lu et al. for trace metal vapors detection (Lu et al., 2010). In this case, Bi powder was simply mixed with carbon ink and the resulting Bi SPE was covered with a 0.1 mm thickness hydrogel layer by immersion in an agarose solution. This latter acted both as solid electrolyte and preconcentration agent, allowing Pb vapor concentrations between 48 and 386 pM to be determined with a LOD around 4.8 pM. Similar results were obtained with Zn and Cd.

CARBON ELECTRODE MATERIALS

Nowadays, a particular attention is paid to more ecofriendly solid electrodes. GC or graphite felts are the easiest carbon based material to obtain (structured with sp^2 carbon atoms). Alternatively, Boron doped diamond (BDD) or diamond like carbon (DLC) with sp^3 carbon atoms structure are very interesting for their specific properties (McCreery, 2008).

Graphite

Graphite felts are porous electrodes made of 10 μm diameter carbon fibers. They generally present a high specific surface area (around 1200 $\text{cm}^2 \text{g}^{-1}$) and a high void volume (around 90%) which enhances hydrodynamics, thus improving the LOD of the resulting sensor. Feier et al. exploited these particular properties to elaborate a flow sensor for Zn^{2+} trace analysis (Feier et al., 2012). A LOD of 500 nM was reached by LSASV with a 5 min duration cathodic preconcentration step. Nevertheless the selectivity toward Zn^{2+} detection was strongly affected by several interfering species such as Fe^{3+} , Cu^{2+} , Co^{2+} , and Ni^{2+} . Nasraoui et al. elaborated a Pb sensor by using a similar flow cell and the same electrochemical method (Nasraoui et al., 2009). A LOD as low as 1 nM was obtained for an overall analysis time of 11 min.

In order to increase the sensitivity, Khadro et al., elaborated a SPE involving active surface carbon deposit (1 mm diameter) for the assay of Hg^{2+} and Ni^{2+} (Khadro et al., 2009). Using DPASV as mutual detecting mode, the sensitivity was respectively 10 and 20 times higher than those obtained with a traditional batch electrochemical cell including a three times higher diameter working electrode. Consequently the LODs decreased to 0.15 and 2.56 nM for Hg^{2+} and Ni^{2+} , respectively. Despite satisfactory performances, the main drawback concerned electrode fouling which obliged the regular regeneration of the surface between analyses and reduced the electrode life-time: Nasraoui et al. observed a decrease about 10 and 20% of the electrochemical signal after the second and third regeneration, respectively (Nasraoui et al., 2009).

Amorphous nitrogenous carbon thin film (a-CN_x)

a-CN_x thin film is a carbon-based material (around 80% sp^2 carbon) including nitrogen atoms. It is synthesized by a

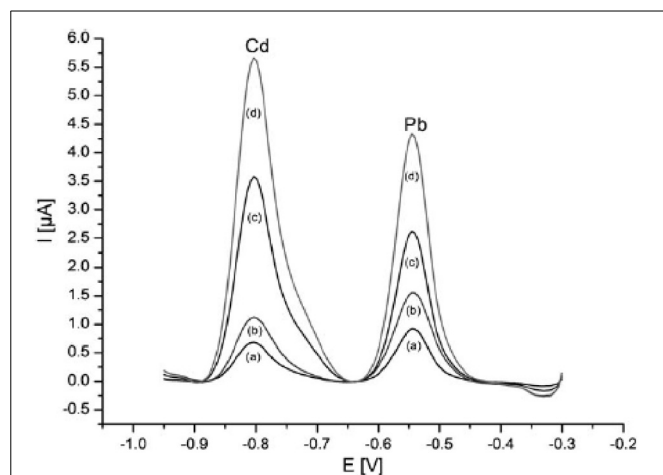
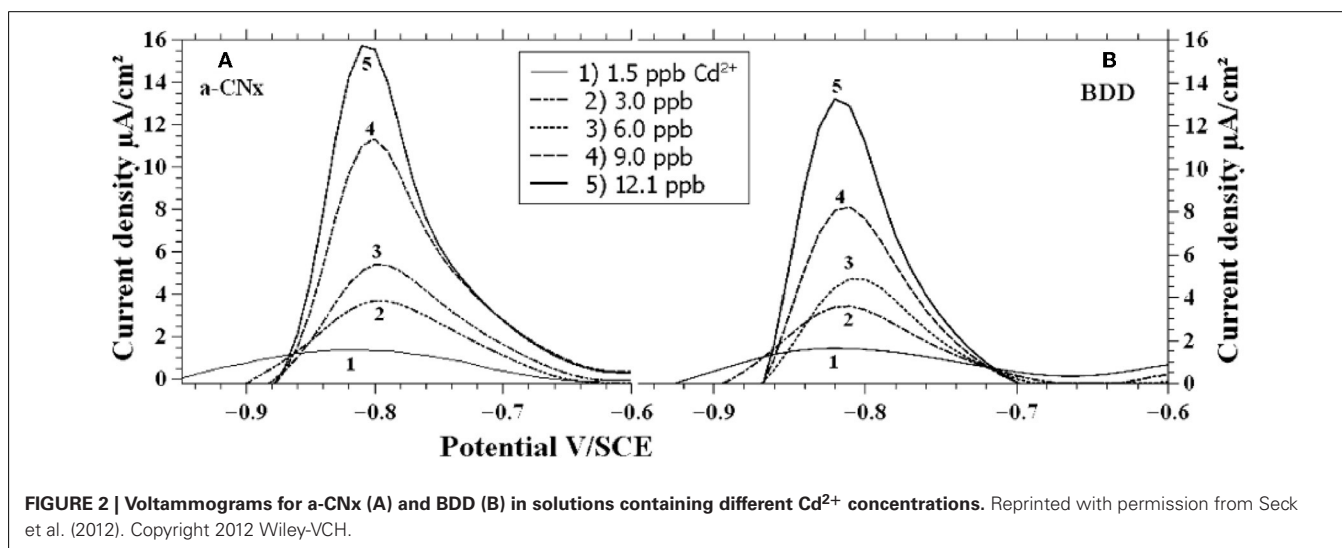


FIGURE 1 | DPASV voltammograms for increasing level of Cd and Pb in the 20–130 ppb range on porous Bi film (gold substrate) electrode.

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radio-frequency magnetron sputtering technique. The amount of nitrogen incorporated into the film can be controlled by the composition of the plasma (N₂/Ar ratio) used for the deposition. The main property of a-CNx electrodes concerns their broad electrochemical window, which makes it particularly suitable for electro detection of many species. This was exploited by Seck et al. for the simultaneous assay of Cd²⁺ and Cu²⁺ (Seck et al., 2012) (Figure 2). The presence of Cu modified the peak current related to Cd²⁺ as compared to Cd²⁺ detection in Cu-free solution. Anyway the a-CNx electrode allowed the detection of 2 ppb of Cd²⁺ with concentration of Cu²⁺ up to 140 ppb.

Diamond like carbon

Diamond like carbon (DLC) is a carbon-based material containing a mixture of sp² (graphite) and sp³ (diamond) carbon phases. Several methods have been developed to produce DLC films: plasma enhanced chemical vapor deposition (CVD) techniques, ion beam, filtered cathodic vacuum arcs. DLC exhibits some unique properties, such as high elastic modulus, high mechanical hardness, very low surface roughness and chemical inertness. Khadro et al. used DLC doped with boron to improve its conductivity (Khadro et al., 2011). The resulting electrode was exploited for the simultaneous assay of many heavy metals in water, namely Cd²⁺, Pb²⁺, Ni²⁺, and Hg²⁺, in concentration ranges up to 200 nM. LODs of 8.9, 4.8, 34, and 5 nM were reached, respectively.

Boron doped diamond

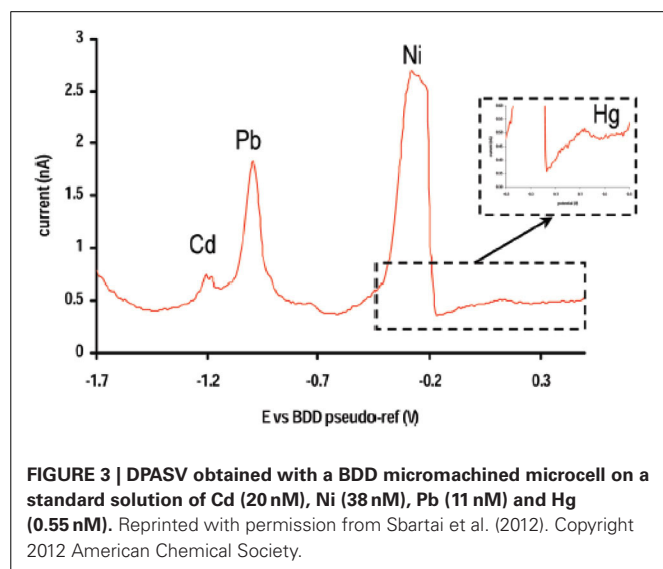
Boron doped diamond (BDD) is the most recent carbon-based material used for electroanalytical purpose. Diamond films can be deposited using CVD systems involving activation of gases by either microwave plasma or a hot filament. Traditionally electrical conductivity of diamond films is obtained through doping with boron (p-type behavior). The advantages of such material are manifold compared to previous carbon-based ones: BDD has an extremely high chemical stability, presents a wide potential window in aqueous media (−1.35 to +2.3 V/NHE at 0.1 mA cm^{−2} in 0.5 M H₂SO₄) and generates a low background current. Moreover it is extremely resistant to fouling phenomena, thus making its

surface state very reproducible with time. Unlike graphite felt, BDD exhibits however lower specific area. In order to increase the sensitivity two major ways have been envisaged. In the one hand, Le et al. associated a BDD electrode with a microelectrodialyser as a preconcentration step (Le et al., 2012). The corresponding device allowed the assay of Pb²⁺ ions with a linear concentration range between 96 and 490 nM and a LOD of 19 nM. The same analytical device was used for the simultaneous detection of Zn, Cd, Pb, and Cu (El Tall et al., 2007). Quantification was possible for the first three heavy metals but the presence of Cu caused interferences. Compared to GC, BDD electrode exhibited an enhanced sensitivity (3 or 5 times) and a longer lifetime in real samples (El Tall et al., 2007). In the other hand, Sbartaï et al. developed a new electrochemical microcell micromachined by a femtosecond laser for the simultaneous detection of Cd, Ni, Pb, and Hg (Sbartaï et al., 2012) (Figure 3). Reduction of the electrode size resulted in mass transport amplification. LODs of 0.4, 6.8, 5.5, and 2.3 nM were thus obtained, respectively. Quantitative results were recorded for concentrations up to 200 nM. These performances are comparable to those obtained on DLC by Khadro et al. for Pb²⁺ and more accurate for Hg²⁺, Cd²⁺, and Ni²⁺ (Khadro et al., 2011). However, a non-linear calibration curve for Hg was obtained in the former paper, which can be explained by the presence of Cl[−] ions in the electrolyte solution, leading to Hg₂Cl₂ formation at the electrode surface.

New carbon materials like a-CNx, DLC or BDD which exhibit high chemical stability and offer a wide potential window hold significant promises for electroanalytical applications. Moreover, there is a wealth of opportunities for nanoscale electrochemical devices based on carbon materials. Nevertheless, interceptions between cations go through either the development of specific calibrations or the chemical modification of the electrode.

ION SELECTIVE ELECTRODES

Ion-selective electrodes (ISEs) are potentiometric sensors that involve a selective membrane which minimizes matrix interferences (Bobacka et al., 2008). The response of these sensors is based on an equilibrium state complexation reaction between the



analyte and the probe with kinetic properties strongly depending on the membrane composition. The potentiometric measurement as well as the nature of the molecular interactions generally allow LODs around $1 \mu\text{M}$ to be reached and a linear concentration range from 10^{-5} to 10^{-2} M in a pH window from 3 to 6. Innovative changes have been made in recent past years to improve these analytical performances. In this context several functionalized materials have been used, including glass, liquid or polymer membranes. In the latter case an ionophore is generally used as the sensing platform. In this way, Yaftian et al. first synthesized phosphorylated calix[4]arene coated on a graphite electrode for the assay of Pb traces (Yaftian et al., 2006). This sensor exhibited a particularly quite long lifetime (up to 8 weeks) and a relatively short response time (17 s). This latter was significantly shortened to 7 s by using hexahomotrioxacalix[3]arene as the ionophore, due to fast exchange kinetics complexation-decomplexation processes (Yaftian et al., 2007). Furthermore the concentration range covered four decades (between 10^{-8} and 10^{-4} M). The specificity of this ISE toward Pb^{2+} was successfully tested in synthetic solution in the presence of 22 interfering species. Nevertheless no measurement was done in real sample water. In both cases, the electrode was also used in potentiometry for the titration of Pb^{2+} solution using a standard EDTA solution.

Glassy materials represent a good alternative, particularly in micro-sensor fabrication. Beyond the advantages of all solid state devices, vitreous materials are well-suited for the production of homogeneous thin layers which allow potentiometric measurements to be done despite their poor conductivity. Thereby, several types of solid state membranes have been synthesized like the widely used chalcogenide one, in which the conduction over the membrane is provided by halides or metallic ions. Chalcogenide glasses exhibit better chemical durability in acidic media, and in many cases, afford better selectivity and reproducibility than arene ionophores. Several French research teams have investigated such ISEs toward Cu(II) determination. Cali et al. used

chalcogenide glassy-crystalline Cu-As-S alloys (Cali et al., 2002). The resulting sensor exhibited a very short response time (1–3 s) and a LOD close to 10^{-6} M with a linear concentration range between 10^{-6} and 10^{-2} M. These results are available within a pH range from 2 to 6. Similar analytical performances were obtained by Essi et al., with a Cu-Ag-S thin film for the assay of Cu(II) and Ag(I) ions (Essi and Pradel, 2011a). They were satisfactorily compared with those obtained by ICP-MS in real samples. Furthermore the specificity of the sensor was not damaged by the simultaneous presence of Ca^{2+} , Mg^{2+} , Pb^{2+} , Cd^{2+} , and Zn^{2+} (Essi and Pradel, 2011b). Mear et al. investigated a thin film of $\text{Ge}_{28}\text{Se}_{60}\text{Sb}_{12}$ chalcogenide glass including Cu in order to quantify Cu(II) ions (Mear et al., 2005). A linear range was found between 10^{-5} and 10^{-3} M with a LOD of $3 \mu\text{M}$. The concentration range was enlarged between 10^{-6} and 10^{-2} M for Cd(II) ions assay by coupling the membrane electrode with a pre-concentration module.

CHEMICALLY-MODIFIED ELECTRODES

From the analytical point of view, the wide and increasing success of chemically modified electrodes (CME) may be explained by the offered possibility to purposely design the surface of conventional electrodes. By combining the intrinsic properties of the modifier and a given electrochemical reaction, CMEs exhibit significantly improved response compared to unmodified electrodes (Murray et al., 1987; Gilmartin and Hart, 1995; Cox et al., 1996). For heavy metals trace detection, the modification plays a critical role especially during the preconcentration step, by favoring selective and enhanced accumulation, thus leading to higher sensitivity and lower LODs (Arrigan, 1994). During the detection step, the modification also often favors the electron transfer kinetics. The modifier may be a mineral such as silica or clay, a polymer, an inorganic or organic compound or a metal nanoparticle based material. Depending on its nature, the modifier is associated to the electrode by adsorption, covalent binding, coating or even dispersion into a conductive matrix.

Minerals

Minerals such as silica-based materials, clays and zeolithes, are of particular interest for ion exchange voltammetry (IEV) (Wang, 1989). Basically, they act as an ion selective film inside which the analytical target is preconcentrated at open-circuit potential by an exchange process. In a second step, the analyte incorporated within the ion-exchanger film is detected by using an anodic stripping technique (Walcarius, 1998).

In France, the research on silica-modified electrodes with respect to heavy metals assay is mainly represented by the group of Walcarius. In 1997 this group published the first report dealing with silica-modified carbon paste electrode (SMCPE) devoted to electroanalysis, with Cu(II) as the analytical target (Walcarius and Bessiere, 1997). By using a 10 min preconcentration step and SWASV, a LOD of 2 nM was reached. This SMCPE exhibited a good reproducibility since up to 30 detection procedures were performed over a period of a week without any noticeable loss of sensitivity. However, this system suffered the classical drawback of CPE, namely a gradual dissolution process. In this particular example, another severe drawback was the necessary use of an

ammonia medium in order to ensure Cu(II) accumulation via its interaction with surface silanolate groups. This pioneering work has been later extended to various silica-based materials and the influence of interfering species has been studied (Walcarius et al., 1999a). Clearly, this SMCPE failed at high ionic strength, since important cations concentration resulted in competition for the ion-exchange sites. A very similar study has been reported with Hg(II) as the analytical target (Walcarius et al., 2000). Using the same procedure and materials, Hg(II) has been found to suffer no influence of ionic strength and no particular interference with other metallic species, even Ag(I). This result has been explained taking into account the formation of soluble Hg(II) hydroxides in the experimental conditions used.

To overcome the drawback of adding a complexing agent in order to ensure metal accumulation, the group of Walcarius has developed several organically modified silica CPEs. Aminopropyl groups (Walcarius et al., 1999b; Etienne et al., 2001) or a carnosine dipeptide (Sayen et al., 2003) have been co-condensed with silane precursors to afford the desired functionalized silica materials. Cu(II) was detected with similar LODs to those reported in the former study (Walcarius and Bessiere, 1997) without adding anything to the accumulation medium. The aminopropyl-grafted silica CPE was successfully tested for Cu(II) detection in laboratory tap water (Etienne et al., 2001). Cu(II) suffered important competition from Co(II), Ag(I), and Hg(II) for the binding sites of the carnosine-modified silica CPE, thus limiting the practical usefulness of this sensor in real media (Sayen et al., 2003).

An interesting alternative to CPE has been proposed later by using silica films coated onto Au (Walcarius and Sibottier, 2005) or GC (Etienne et al., 2007; Sanchez and Walcarius, 2010) electrodes. Whereas these films have been prepared most of the time by a classical surfactant-templated synthesis (Etienne et al., 2007; Sanchez and Walcarius, 2010) an original electrochemically-induced sol-gel deposition has been

also reported (Walcarius and Sibottier, 2005) (**Figure 4**). All these films were functionalized either by thiol or amine groups. Cu²⁺ (Walcarius and Sibottier, 2005), Ag⁺ (Etienne et al., 2007) and Hg(II) (Sanchez and Walcarius, 2010) were the analytical targets, and the LODs obtained were 40, 6, and 24 nM respectively.

From a more general point of view, all these studies on silica-based modifiers proved that the key feature with respect to analytical performances, and particularly sensitivity, is the analyte diffusion inside the porous structure of silica, thus making porosity a more predominant factor than the amount of surface silanol groups (Walcarius et al., 1998). Thus, mesoporous silica-based materials, which exhibit well-defined three-dimensional structures, appear to be much more adapted for heavy metal preconcentration than amorphous ones (Walcarius et al., 2003), whatever they are functionalized (Ganesan and Walcarius, 2008) or not (Walcarius et al., 1998). When functionalized, the amount of organic groups is also of importance, its effect on the analyte preconcentration passing by a maximum, since too much organic groups lead to a decrease in pore size which hampers the accessibility of the binding sites (Etienne et al., 2007; Ganesan and Walcarius, 2008; Sanchez and Walcarius, 2010).

Clays may also be used to perform IEV. These minerals exhibit relatively large specific surface areas and ion-exchange properties associated to an ability to sorb and intercalate many compounds. In a very close approach to what has been reported for silica-based material, their surface may be functionalized by organic groups (Navratilova and Kula, 2003), affording the possibility to tune charge selectivity of clays in IEV (Tonle et al., 2004).

In France, the group of Walcarius explored the potentialities of clays functionalized by organic groups and mixed with CPE toward Hg(II) (Tonle et al., 2003, 2005) and Pb(II) (Tonle et al., 2011) determination. With respect to Hg(II), the comparison of thiol-functionalized vs. amine-functionalized clays showed that the former one exhibited a better LOD (68 and 87 nM,

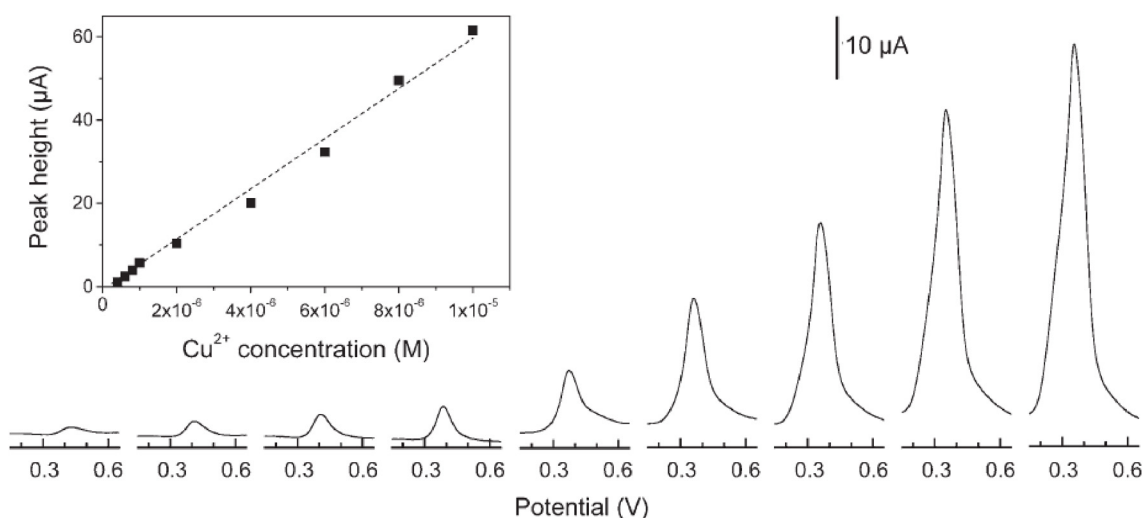


FIGURE 4 | Typical DPASV and calibration (inset panel) curves obtained for Cu²⁺ using a 10% amine functionalized silica film deposited on gold. Reprinted with permission from Walcarius and Sibottier (2005). Copyright 2005 Wiley-VCH.

respectively, using DPASV with 10 min accumulation), in accordance with the greater affinity of Hg for sulfur groups (Tonle et al., 2005). Thiol-functionalized clays have been also coated as thin films onto GC (Tchinda et al., 2007, 2009). The LOD has been greatly improved since a 6 nM value was reached using DPASV with only 3 min accumulation, the linear range being from 50 to 800 nM (Tchinda et al., 2007). It has to be noticed that increasing the accumulation time allowed another linear range to be observed from 4 to 20 nM together with a remarkable LOD of 0.5 nM (Tchinda et al., 2007).

Macrocyclic compounds

Macrocyclic compounds have received much attention in the last two decades due to their particular three-dimensional shape which provides a suitable cavity for selective complexation of heavy metals (Kolthoff, 1979). French groups particularly focused their research on cyclams (Goubert-Renaudin et al., 2009a,b; Nasraoui et al., 2010a,b), crown-ethers (Parat et al., 2006; Betelu et al., 2007) and calixarenes (Dernane et al., 2013; Rouis et al., 2013).

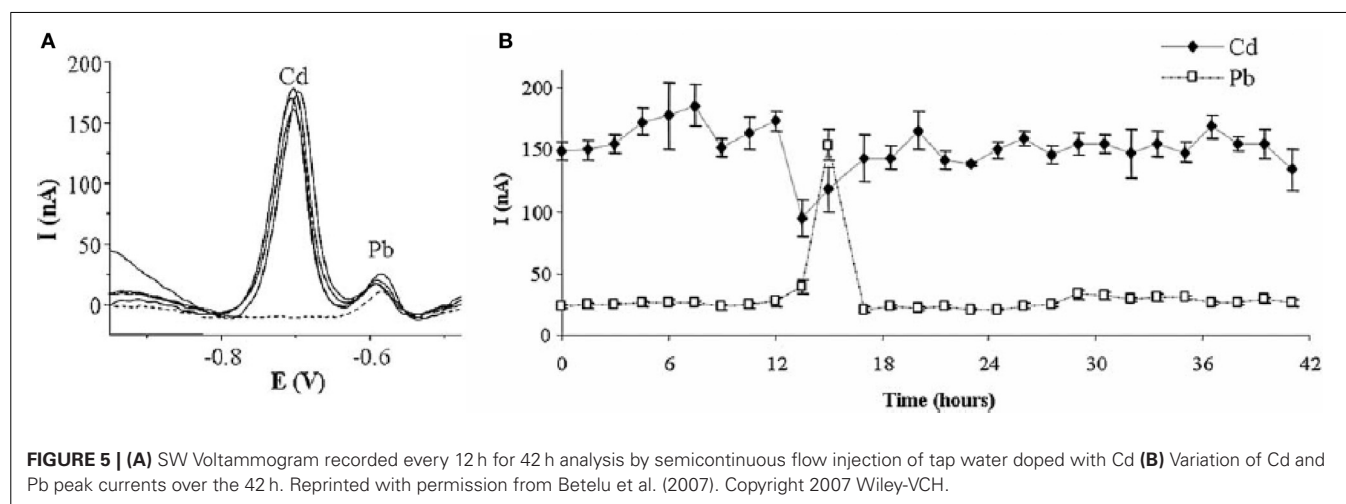
With respect to these latter compounds, Dernane et al. proposed to detect Cd^{2+} thanks to a *p*-tert-butylcalix[8]arene membrane deposited onto Au electrode (Dernane et al., 2013). In this work the metal cation coordination was favored by the presence of the four ketone groups on the calixarene, thus allowing a 0.1 μM LOD to be reached. However the results seemed a little bit cautious since detection was performed simply by CV without further preconcentration step. Moreover, the variation of peak current density as a function of the logarithm of metal concentration was proposed as a calibration plot, which makes non-sense with respect to classical equations of CV.

Rouis et al. have built an impedancemetric sensor dedicated to Hg^{2+} detection by immobilizing a β -ketoimine calix[4]arene derivative in a conducting poly(*p*-phenylene vinylene) membrane deposited onto indium-tin oxide (ITO) electrodes (Rouis et al., 2013). The main originality of this work was the study of light excitation effect of the β -ketoimine calix[4]arene while optimizing detection parameters. Charge transfer processes at the electrode/electrolyte interface were found to be improved under light excitation, thus providing an enhancement of the sensitivity

toward Hg^{2+} . The best results were obtained under blue light emission, providing a charge transfer resistance $R_{ct} = 4.47 \text{ k}\Omega$ and a linear range from 0.1 nM to 0.5 μM .

Parat et al. reported the use of a thin-film mercury-coated screen-printed carbon electrode covered by a crown-ether based membrane for the determination of Cd and Pb (Parat et al., 2006). The crown-ether membrane aimed at protecting the active surface from interferences during the analysis. The size of crown-ethers cavity has been proved to impact the performances of the electrode. The best results have been obtained for the smallest crown-ether, because its cavity size fitted well to metal ions radii, although Pb was a bit favored. LODs of 6 nM and 8 nM for Cd^{2+} and Pb^{2+} , respectively, have been reached by using LSASV with 2 min preconcentration. Tests were successfully conducted on river water and soil solution extracts. This system was then applied in a later work to the semi-continuous monitoring of Cd and Pb in tap water by FIA and afforded reproducible results for 42 h (Betelu et al., 2007) (Figure 5).

Goubert-Renaudin et al. have developed a new family of functionalized cyclams they grafted onto silica materials and mixed to CPE (Goubert-Renaudin et al., 2009a,b). In the first work dedicated to Cu(II) detection, the functionalization aimed at stabilizing cyclams on silica in order to improve the sensor lifetime. However, increasing the number of functionalizations per cyclam lead to an increase in cycle rigidity, which has been correlated to lower performances with respect to Cu(II) uptake (Goubert-Renaudin et al., 2009a). The modified CPE afforded good stability, with a 7% relative standard deviation for 45 measurements and was successfully tested on tap water. In a second work, dealing with Pb(II) determination, the aim of the functionalization was to improve the analyte preconcentration step by favoring complexation (Goubert-Renaudin et al., 2009b). Thus, the inverse trend was observed, i.e., the more functionalized the cyclam, the better the performances. The best system allowed LODs down to 2.7 nM to be reached. Among potential interfering species, only Hg(II), Cd(II), and Cu(II) gave rise to significant loss of signal, mainly because of competition for the binding sites. The group of Geneste also used such kind of cyclams to functionalize graphite felt electrodes (Nasraoui et al., 2010a,b). Accumulation of Pb(II) at open-circuit potential by flowing the



aqueous solution to analyze, followed by LSASV allowed a 25 nM LOD to be reached. However, this system exhibited relatively poor stability since a 20% decrease of the response was reported after regeneration.

Polymers

Electroactive surface modification by means of polymer deposition or electrodeposition represents a broad research field, leading to numerous papers every year. With respect to trace metals analysis, polymer films allow the immobilization on the electrode of a large number of ligands which may complex metal ions to be accumulated (Trojanowicz, 2003; El Kaoutit, 2012; Li et al., 2012). The polymers used for surface modification may be natural or prepared purposely by chemical synthesis. However, with respect to French groups' research activities, no work was found dealing with natural polymers dedicated to trace metal detection. The only papers found in the literature were about polysaccharides (Crini, 2005) and chitosan (Vieira et al., 2011) and concerned heavy metals removal.

Concerning chemically synthesized polymers, the group of Moutet has developed two different groups of substituted polypyrrole derivatives for trace metal determination. The first one is based on "poly(pyrrole-EDTA like)" and takes advantage of the well-known complexing properties of EDTA to improve metal preconcentration (Heitzmann et al., 2007; Buica et al., 2009a,b). These studies were devoted to the assay of Cu(II), Pb(II), Cd(II), and Hg(II) (Figure 6). In the first two ones, the selectivity of the modified electrode has been tuned by varying the accumulation time and the pre-structuration of the polymer. The film thickness was also proved to influence the selectivity. However, the electrode was insensitive to Cd(II) whatever the conditions adopted. To overcome this problem, Heitzmann et al. have chosen an imprinted polymer strategy: the polymer was electrodeposited in the presence of Cd(II) ions which were then removed from the metallopolymer film (Heitzmann et al., 2007). The resulting functionalized electrode was thus able to detect Cd(II) as well as the other three metal cations. By introducing 4 pyrrole fragments on the same EDTA skeleton instead of only two in the former studies, an enhanced stability and a better controlled dimensionality was conferred to the polymer, thus making the sensor response independent on the film thickness (Buica et al., 2009a). The global complexing capability of the polymer was also greatly improved by the presence of 2 amine and 4 amide coordinating groups per monomer unit. The second group of polymers developed by the group of Moutet is based on poly(pyrrole-malonic acid) (Heitzmann et al., 2005; Pereira et al., 2011). Here, the analyte complexation occurred with the anionic form of malonic acid, which is known to easily coordinate various metal ions. This sensor was also tested with Cu(II), Pb(II), Cd(II), and Hg(II). It allowed LODs around 10^{-10} M to be reached for each metal cation, and exhibited good stability since the same current response was obtained for 2 assays at a 3 weeks interval using the same electrode stored without any particular precaution.

Another kind of polymer based on styrene units gave rise to one report by Moutet (Rivas et al., 2006). Styrene was copolymerized with acetamide acrylic acid or itaconic acid. These latter are hydrophilic whereas styrene is hydrophobic,

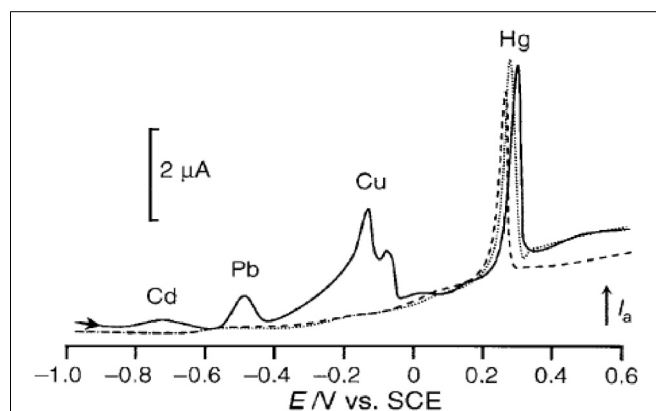


FIGURE 6 | DPV curves recorded at a poly(EDTA-like) film modified carbon electrode in acetate buffer containing Hg(OAc)₂ (dotted line), Cu(OAc)₂, Cd(NO₃)₂, Pb(NO₃)₂, Hg(OAc)₂ (full line). Reprinted with permission from Buica et al. (2009b). Copyright 2009 Wiley-VCH.

thus providing interesting mixed properties to the resulting copolymers. The incorporated carboxylate groups were used for the accumulation and detection of Pb²⁺ but the system exhibited poor performances since the response was linear only in the narrow range from 10^{-5} to 10^{-3} M.

Bessbousse et al. proposed a more sophisticated system based on a nanoporous β -poly(vinylidene fluoride) (β -PVDF) membrane (Bessbousse et al., 2011). The nanopores were further functionalized by track-etched poly(acrylic acid) (PAA), and thin porous Au films were sputtered on each side of the membrane. This very sophisticated electrode has been proved to detect Pb²⁺ but no very clear analytical results were provided.

Polythiophene also gave rise to one report (Zejli et al., 2007). The polymer was electrodeposited on a Pt electrode by cyclic voltammetry and used to detect Ag(I) by DPASV taking advantage of the inductive effect of the C-S dipole of thiophene units. However, the linear range was found to be very narrow, from 0.65 to 9.3 μ M.

A contribution from the group of Noguet has also to be noticed (Yasri et al., 2011). In this original work, a graphite electrode coated by a 3,4-poly(ethylenedioxythiophene):poly(styrene sulfonate) [PEDOT:poly(styrene sulfonate)] copolymer was used to detect Pb²⁺ by chronoamperometry at -0.35 V. The linearity range was from 2 to 100 nM and the LOD was 0.19 nM for 30 s accumulation at -0.65 V. The system exhibited good stability since only a slight decrease was noticed after 11 days. It was also successfully tested for the determination of Pb²⁺ in different vegetables extracts.

In order to improve the detection limit the group of Chevalet exploited the high resolution power of a multi-pulse electro-analytical method, namely multiple square wave voltammetry (MSWV) (Fatouros et al., 1986; Krulic et al., 1990). MSWV is based on the superimposition of several pairs of opposite pulses of constant amplitude on each step of a staircase waveform. However MSWV differs from previously described DAPV by the electrode response: instead of the double cathodic and anodic

current recorded in the latter case, MSWV response results in the integration of the successive currents. The MSWV-DD (DD for double differential) technique was used in combination with a Nafion-coated electrode for the determination of trace species like methylmercury (Moretto et al., 1999) and Fe (Ugo et al., 2001). The perfluorinated cation exchanger Nafion was used to preconcentrate the analyte and was simply deposited on a GC disk by droplet evaporation. The detection capabilities of this polymer-coated electrode combined to the sensitive MSWV-DD method allowed a calculated LOD down to 45 pM to be reached for methylmercury (Moretto et al., 1999).

Nano-scaled materials

During the last two decades, nano-scaled materials have aroused a great interest with respect to analytical applications due to their specific physico-chemical properties (Murray, 2008). Improvements resulting from those nanomaterials for electroanalysis are manifold: enhanced diffusion of electroactive species, higher effective surface area of nanoparticles (NPs), electrocatalytic and conductive properties, improved selectivity and higher signal-to-noise ratio. With respect to trace metal analysis, gold nanoparticles (AuNPs) are the most commonly used material (Lin et al., 2011; Liu et al., 2011). They can be obtained either by chemical or electrochemical ways.

The French contribution to this topic is very recent, and the corresponding works all considered the structuration of the nanoparticle-based modified electrode to be a key feature with respect to analytical performances.

Ottakam Thotiyl et al. designed a multilayer arrangement of citrate-capped AuNPs immobilized by a thiol group onto a Au electrode for the detection of As(III) (Ottakam Thotiyl et al., 2012). The anionic AuNPs were deposited layer-by-layer alternatively with cationic polyelectrolyte to afford a layered nanocomposite film. As(III) was detected by its electrocatalytic oxidation using DPV without any accumulation step, and a LOD of 0.48 μM was reported. This study evidenced a strong correlation between the amperometric response and the number of layers of the nanocomposite film.

Our group has developed a Hg(II) sensor based on GC electrode functionalized by electrodeposited AuNPs and has studied the influence of the electrodeposition method on the analytical performances (Hezard et al., 2012a,b). Namely, cyclic voltammetry, chronoamperometry and potentiostatic double-pulse were used. It was shown that both the electrodeposition mode and the charge used for the Au precursor reduction had a dramatic influence on the size and density of AuNPs (Figure 7). These latter two parameters were strongly correlated to the analytical performances: the best results were obtained for dense deposits of small NPs (Hezard et al., 2012b). By using a 5 min accumulation time and SWASV, a LOD of 0.4 nM was reached.

An important development in the frame of nano-scaled materials concerns single (SWCNTs) or multi-walled carbon nanotubes (MWCNTs) since their discovery in 1991 (Ijima, 1991). Their unique structure offers very interesting properties such as high specific surface area, high chemical stability, good electrical conductivity and adsorption capacity, which give rise to

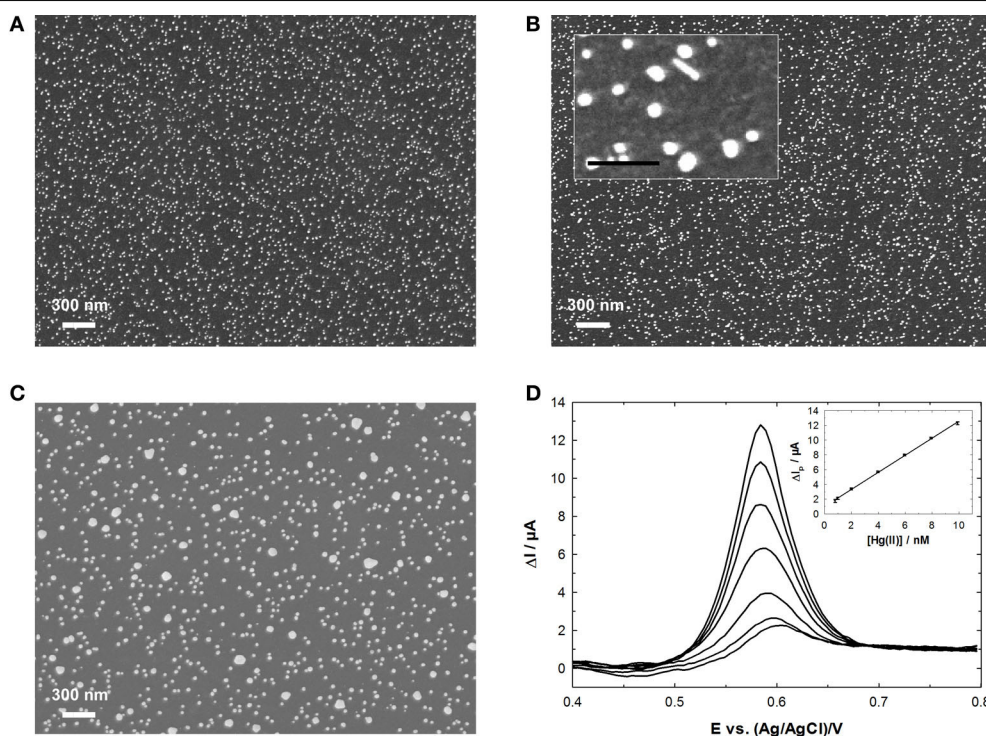


FIGURE 7 | AuNPs electrodeposited onto GC from a 0.25 mM HAuCl_4 solution using: (A) chronoamperometry; (B) potentiostatic double pulse; (C) cyclic voltammetry. (D) SWASV response and calibration curve obtained

in the Hg(II) concentration range 0.8–9.9 nM using electrode (A). Adapted from our own results published in Hezard et al. (2012b), Copyright 2012 Elsevier.

wide applications in electronics, composite materials, energy storage, and of course sensors (Fam et al., 2011). With respect to this latter domain the association of various CNT functionalization procedures, including organic (polymers, proteins...) or inorganic (metal nanoparticles, metal oxides...) modifiers and several transducing modes allowed the elaboration and development of chemical, gas, humidity, biomedical or environmental sensors which have been the subject of several reviews (Jacobs et al., 2010; Vashist et al., 2011; Gao et al., 2012). Extensive international researches have been made to assay heavy metals by using unmodified (Yue et al., 2012) or CNTs modified either with cysteine (Morton et al., 2009), thiacalixarene (Wang et al., 2012), Sb nanoparticles (Ashrafi et al., 2014) or with imprinted polymeric nanobeads (Rajabi et al., 2013), most of the time associated with ASV. They allowed Pb, Hg, Cu, Cd, or Zn to be detected down to 3 nM and in linear concentration ranges up to 7 μ M. Surprisingly, to the best of our knowledge, no French research team has exploited CNTs for heavy metals detection yet. Interest has mainly focused on micro and supercapacitors (Ghimbeu et al., 2011), batteries (Carn et al., 2013) and energy storage devices (Sathiya et al., 2011) or biofuel cells (Both Engel et al., 2013; De Poulpiquet et al., 2013). Concerning sensors works essentially dealt with gas sensors (Bondavalli, 2010) and above all enzymatic (Singh et al., 2013), RNA (Tran et al., 2013) and DNA (Zhang et al., 2011)-based electrochemical biosensors.

Biosensors

In general terms, a biosensor is defined as an analytical tool associating a bioactive compound (mono- or multi-enzyme system, antibody, microorganism) which can specifically recognize species of interest and a transducing element (Frew and Hill, 1987). Thus, it may be viewed as a particular kind of CME in which the modifier is a biological element. Enzymes are known as good modifiers with respect to heavy metals since these latter often strongly inhibit enzymatic reactions (Dennison and Turner, 1995).

The group of Chovelon reported several works about conductometric biosensors based on *Chlorella vulgaris*, a microalgae the cell walls of which bear enzymes such as alkaline phosphatases and esterases (Chouteau et al., 2004, 2005). In both papers, the microorganism was immobilized onto interdigitated Pt electrodes using bovine serum albumin and glutaraldehyde as a crosslinker. In the first work, Cd^{2+} was detected as low as 8.9 nM but at least one hour was needed to obtain such a result. The second study (Chouteau et al., 2005) provided several improvements since both Cd^{2+} and Zn^{2+} were detected with a 89 nM and 0.15 μ M LOD, respectively, but with a significantly lower exposure time (ca. 30 min). It has to be noticed that bioassays were found to reach a similar LOD to the biosensor but only after 4 h exposure time, illustrating the wise enzyme immobilization strategy used. Finally, an originality of this latter work was that the biosensor was also capable to detect pesticides, taking advantage of the fact that esterases were selectively inhibited by these organic compounds whereas alkaline phosphatases were not affected.

Tekaya et al. have also reported a conductometric biosensor based on alkaline phosphatase from the cyanobacterium *Arthrospira platensis* (Tekaya et al., 2013) deposited on the ceramic

part of interdigitated Au electrodes. In this case, inhibition measurements were performed after 24 h incubation, allowing LOD down to 10^{-20} M to be reached for both analytical targets, namely Cd^{2+} and Hg^{2+} . Despite this very appreciable LOD, no information was provided about sensor lifetime. This is mainly due to the fact that the authors considered their study as a proof of concept aiming at providing a global response to the presence of heavy metals.

A last conductometric biosensor was reported 2 years ago by Soldatkin et al. (2012). This sensor consisted in a three-enzyme system prepared by mixing invertase, mutarotase and glucose oxidase with bovine serum albumin. Although a bit complicated, the sensor exhibited good sensitivities toward Hg^{2+} and Ag^{+} without experiencing any interference from organic compounds. After a relatively short incubation time (ca. 10–20 min), the LOD were found to be 25 and 100 nM for Hg^{2+} and Ag^{+} , respectively. It has to be noticed that this biosensor was very selective since Cd^{2+} , Zn^{2+} , Ni^{2+} , Pb^{2+} , Cu^{2+} , and Co^{2+} did not affect the enzymes activities. Interestingly, the authors have investigated a reactivation procedure: dipping the biosensor into EDTA or cysteine solution for 45 min allowed a significant reactivation of the enzymes together with the identification of the inhibitor metal, since EDTA reactivated Ag^{+} inhibition only whereas cysteine was selective to Hg^{2+} inhibition.

The group of Cosnier proposed an amperometric sensor toward Hg(II) based on the same three enzymes which were entrapped in a clay matrix deposited on Pt electrode (Mohammadi et al., 2005). The electrochemical response was provided by the oxidation of the enzymatically produced hydrogen peroxide. After 20 min incubation, Hg(II) was detected in the range 10 nM to 1 μ M. It has to be noticed that several Hg species have been tested, such as methylmercury or phenylmercury, all of them leading to invertase inhibition. Depending of the analytical target, the biosensor recovery by using cysteine was more or less effective, but never complete. The system was quite selective to Hg(II) since all metal cations tested induced no interferences except Ag^{+} .

Finally, the group of Burstein has reported 20 years ago a bi-enzymatic system coupled to an oxygen sensor (a Clark electrode) which offered an original solution to the enzyme recovery issue (Gayet et al., 1993). In this work, several enzyme combinations were tested but the most interesting one was that involving L-lactate oxidase and L-lactate dehydrogenase. Actually, the former enzyme was immobilized on the membrane of the oxygen sensor whereas the latter one remained in solution. Since only L-lactate dehydrogenase was affected by the presence of heavy metal cations, the sensor regeneration simply consisted in a renewal of the solution containing this very cheap enzyme. Unfortunately, the interest of this system was limited due to the relatively poor LOD obtained for the different analytical targets.

THE PARTICULAR ISSUE OF TRACE METAL SPECIATION

In addition to the monitoring of total heavy metals concentrations in the environment, speciation analysis provides very useful complementary informations. However speciation analysis often implies the determination of very low concentrations of minor species, typically in the range of nM to pM or even lower. Hence

electrochemistry and especially anodic stripping techniques are particularly well adapted to such kind of analysis.

For instance, Bourgeault et al. studied dissolved Cu speciation and bioavailability in freshwaters by using DPASV, diffusive gradient in thin films and ISE, and compared their results to those obtained by modeling (Bourgeault et al., 2013). It was found that Cu accumulation in aquatic mosses was better correlated to the weakly complexed Cu species measured using DPASV than to free Cu concentration measured using an ISE, thus highlighting the contribution of electrochemical techniques to speciation studies.

In a close approach combining DPASV and conductometry, Terbouche et al. examined the complexation of Zn(II) and Cd(II) by new humic acids from Algeria, these latter class of natural polymers being known to have an influence on heavy metals biogeochemical cycle and bioavailability (Terbouche et al., 2011). Based on the strong complexing capacities evidenced in this work, the authors suggested their humic acids to be used for metal uptake.

A last speciation study combining electrochemical techniques was provided very recently by Rotureau (2014). Here the already discussed "AGNES" technique was used for the determination of free metal in solution while SCP at scanned deposition potential (SSCP) allowed dynamic speciation information to be reached. This latter technique consists in performing classical SCP with varying deposition potentials, and then plotting the transition time as a function of the deposition potential (van Leeuwen and Town, 2002). Rotureau's work dealt with Cd and Pb speciation dynamics in clay minerals, and it was shown that the interaction of Cd with clays may be described as a chemically homogeneous, labile system over a wide pH range whereas strong pH effects were evidenced in the case of Pb.

SSCP has also been used by Parat et al. to study Cd speciation parameters in the presence of several ligands, namely nitrilotriacetic acid (NTA) and 2,6-pyridinedicarboxylic acid (PDCA) (Parat et al., 2011b). By using a Hg film SPE and a 60 s deposition time, a LOD of 2.2 nM was reached and some information upon speciation extracted. In particular, it was demonstrated that Cd-NTA complex is less labile than Cd-PDCA.

It has to be noticed that classical SCP has been used by the group of Riso for the determination of Se(IV) and total Se in seawater on a HgFE (Riso et al., 2004). However in this case, speciation information was reached by tuning the experimental parameters of water pretreatment, the electrochemical ones remaining nearly the same. Thus, Se(IV) concentration was determined by performing SCP on untreated water, whereas total Se was reached by analyzing UV-digested water.

Among all anodic stripping methods, pseudopolarography is particularly well-suited to provide useful information on metal speciation in model solutions and in natural water. This electrochemical technique allows thermodynamic and kinetic properties of the different forms of metal ions to be reached. The current-potential curves obtained, called pseudopolarograms, depict the influence of the metal deposition potential on the peak height, the peak potential or the half-peak width of the subsequent anodic voltammogram. The shifts in peak potentials as well as the changes in the slope of the curve indicate a modification in the reaction kinetics or reversibility. These

evolutions make it possible to discriminate between different fractions of metals, including free or labile species and electroinactive metal complexes, and to detect the presence of complexing ligands at low concentration. Compared to classical linear or pulsed voltammetry, pseudopolarography allows the speciation of trace metals at natural concentration level (generally less than nM). Pizeta et al. have taken benefit of this properties to detect Pb(II) in sediment and interstitial water with an iridium solid microelectrode covered by a thin mercury film (Pizeta et al., 2005). The electrode sensitivity varied between 4 and 20 nA / μ M and its LOD was about 30 nM. The amperometric response was repeatable enough, avoiding memory effect and electrode surface blocking. Particularly the results highlighted the existence of strong complexes in sediment and in non-filtered interstitial water with high pH. More recently this procedure was successfully applied for the study of the interactions of natural (NOM) or dissolved (DOM) organic matter with Cu and Zn (Louis et al., 2008; Nicolau et al., 2008) in seawater sample. The unpolluted marine water containing DOM about 1 mg L⁻¹, measurements were possible in the latter case after concentrating DOM in large water volumes by nanofiltration and reverse-osmosis. Anyway the results gave comprehensive elements on the role of marine DOM in metals speciation: the dependence of pseudopolarograms with pH clearly indicated the differences of DOM sites for Cu and Zn complexation regarding their stability and acidity constants. The main drawback is the time-consuming of the method which requires a set of anodic stripping voltammograms to be recorded for each pseudopolarogram.

To end this section dedicated to heavy metal speciation, let's talk about some works which are certainly not the most original ones in terms of electroanalytical method, but present particularly interesting automated systems. Zaouak et al. recently developed a system for Cd semicontinuous monitoring which allowed the determination of total and bioavailable Cd (Zaouak et al., 2010a,b). The analyses were performed using a Hg film deposited on a SPE, this latter electrode being integrated in a fully-automated device allowing all the operations needed for a complete set of measurements. Thus, a measurement cell, a storage cell and a plating cell for the electrode renewal were comprised in the whole device, together with a hydraulic conveyor which allowed the three-electrode system to move from one cell to another. This automated device was tested on tap water by recording measurements every 160 min for 18.5 h and exhibited a LOD of 1.78 nM. Total and bioavailable Cd were obtained by adjusting the pH value of the treated water.

Another example of automated electroanalytical device was reported by Hezard et al. for Fe(III) and Fe(II) quantification in acid mining drainage water (Hezard et al., 2009). In this particular case, the redox species were concentrated enough to allow direct determination using an unmodified Pt electrode. Moreover, the presence of sulfide anion S²⁻ in acid drainage water induced an important negative shift in potential for O₂ reduction, resulting in a well-separated reduction wave for Fe(III). Thus, both Fe(III) and Fe(II) were determined using a single potential scan without deaerating the solution. An originality of this work was that

both sample renewal and hydrodynamic control were achieved by a fluidic system based on gravity instead of a pump.

CONCLUSION

From an historical point of view, the unique properties of the conventional mercury electrode have been at the origin of considerable efforts with respect to electrochemical methods devoted to heavy metals detection in water. Nowadays the great experience in polarographic techniques coupled with the improvements in data processing for the monitoring of pulsed potential sequences is still encouraging several groups to exploit the background on this electrode. Nevertheless recent trends have focused on mercury-free electrode since this metal will be totally prohibited in the next few years (European Directive 2008/105/EC). Depending on whether the goal is heavy metal pollution detection in industrial waste or drinking water analysis, detection limits in the μM or nM have to be reached, respectively. In the former case ISEs are well-suited due to the simplicity of the potentiometric measurement and the good understanding of the sensing mechanism. On the other hand the detection of trace metals requires more sensitive methods. In that way CMEs represent a good alternative toward expensive, inconvenient analytical methods developed in laboratory. The combination of amperometric transduction with the specificity induced by the modifier makes it possible to propose reagentless sensors exhibiting high sensitivity and good selectivity. Furthermore the large range of available strategies for surface modification allows multielectrodes or micro array sensors to be designed for simultaneous multicomponent analysis in complex media. Finally a new generation of analytical devices called "micro total analysis system" (μTAS) is being developed for the "on line" monitoring of real samples. These devices integrate not only the sensing element but also some complementary actuators (pumps, valves, microfluidic channels) to obtain an all-integrated automatic monitoring system which includes a calibration procedure. A few prototypes have been recently proposed by using microtechnology manufacturing and semi-conductor processing (Jothimuthu et al., 2011; Jung et al., 2011) but are still at the emerging state. Still a good challenge for French research community!!

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Stress and polyamine metabolism in fungi

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Fungi, as well as the rest of living organisms must deal with environmental challenges such as stressful stimuli. Fungi are excellent models to study the general mechanisms of the response to stress, because of their simple, but conserved, signal-transduction and metabolic pathways that are often equivalent to those present in other eukaryotic systems. A factor that has been demonstrated to be involved in these responses is polyamine metabolism, essentially of the three most common polyamines: putrescine, spermidine and spermine. The gathered evidences on this subject suggest that polyamines are able to control cellular signal transduction, as well as to modulate protein-protein interactions. In the present review, we will address the recent advances on the study of fungal metabolism of polyamines, ranging from mutant characterization to potential mechanism of action during different kinds of stress in selected fungal models.

Keywords: polyamines, stress response, polyamine mutants, fungi, metabolism

INTRODUCTION

Sudden changes in the external conditions can directly impact the internal environment of all living organisms, and can disrupt their homeostasis and normal physiology. Therefore, cells have developed complex systems to identify the status of their environment, and rapidly generate defense systems against environmental stress (Gasch, 2007; Lushchak, 2011; Montibus et al., 2013). An effective procedure to obtain information on the mechanisms regulating the stress response is the identification of pathways or specific genes suffering changes in their expression under stress conditions. Accordingly, it has been shown that a large number of genes are affected in their expression when an organism responds to an environmental stress. Many of these genes are conserved among fungi, including those involved in carbohydrate metabolism, protein metabolism, defense against reactive oxygen species (ROS), intracellular signaling, etc. (Giaever et al., 2002; Gasch, 2007). Among the known elements related with stress response are polyamines, as it has been widely demonstrated in plants (Galston and Sawhney, 1990; Alcazar et al., 2010; Gill and Tuteja, 2010a; Gupta et al., 2013). However, in recent years, investigations into the molecular genetics of fungal polyamine metabolism have led to the isolation of mutants altered in this function that show alterations in their response to stress. The importance of polyamine participation in the response to stress has been highlighted by the increasing number of reports describing the changes occurring in polyamine concentrations induced in response to diverse forms of stress. Accordingly, in the present review we describe the recent genetic and molecular evidences illustrating the role of polyamine metabolism in the responses of fungi to stress.

POLYAMINE METABOLISM IN FUNGI

BIOSYNTHESIS OF POLYAMINES

Putrescine, spermidine and spermine are low-molecular-mass aliphatic cations critical to cell survival (Tabor and Tabor, 1984), and the pathways for their biosynthesis have been analyzed in all the kingdoms of living organisms [e.g., see Valdes-Santiago et al., 2012a]. However, there are some basic differences in the distribution of polyamines among them. In general, it is accepted that in fungi, as well as in animals, there is only one mechanism to produce putrescine *de novo*: by decarboxylation of ornithine by the enzyme ornithine decarboxylase (Odc, E.C.4.1.1.17), which is the first and rate-limiting enzyme in the synthesis of polyamines. In most fungi decarboxylation of ornithine is the only route to putrescine synthesis, however, in plants there is an additional route to produce putrescine: the decarboxylation of L-arginine by arginine decarboxylase (for more details of the general pathway in plants refer Alcazar et al., 2010; Gupta et al., 2013). Nevertheless, some authors have reported arginine decarboxylase activity in fungi such as *Ceratocystis minor*, *Verticillium dahliae*, *Colletotrichum gleosporoides*, and *Gigaspora rosea*, suggesting that both, arginine and ornithine decarboxylase enzymes could be involved in putrescine biosynthesis (Khan and Minocha, 1989; Weerasooriya et al., 2003; Sannazzaro et al., 2004). Putrescine is converted to spermidine by the addition of an aminopropyl group. S-adenosylmethionine decarboxylase (Samdc; E.C.4.1.1.50) is responsible for the formation of the donor of the aminopropyl group, decarboxylated S-adenosylmethionine (dcSAM), and spermidine synthase (Spe, E.C.2.5.1.16) is the transferase of the aminopropyl group from dcSAM to putrescine. Finally, spermidine is converted to spermine by a similar reaction, resulting in the transfer of an

aminopropyl group to spermidine by spermine synthase (Sps; E.C.2.5.1.22) (see **Figure 1**). It should be noticed that this last step does not occur in most fungi, which accordingly contain only putrescine and spermidine (Nickerson et al., 1977). An ortholog of the gene encoding Sps is found only in the subphylum Saccharomycotina of the Ascomycota phylum which include few human pathogens and at least 10 phytopathogenic species (Suh et al., 2006; Pegg and Michael, 2010). Also interesting is to mention that *SPE* gene is present in members of Basidiomycota subphyla as a bifunctional gene encoding spermidine synthase and saccharopine dehydrogenase, the last enzyme involved in lysine biosynthesis (Leon-Ramirez et al., 2010). Regarding polyamine distribution, in general, eukaryotes have low putrescine content and a high content of spermidine and spermine, while prokaryotes have a higher concentration of putrescine than spermidine (Manni et al., 1987). A difference between fungi and plants is the presence of thermospermine, an isomer of spermine that has not been found in fungi (Fuell et al., 2010; Takano et al., 2012)

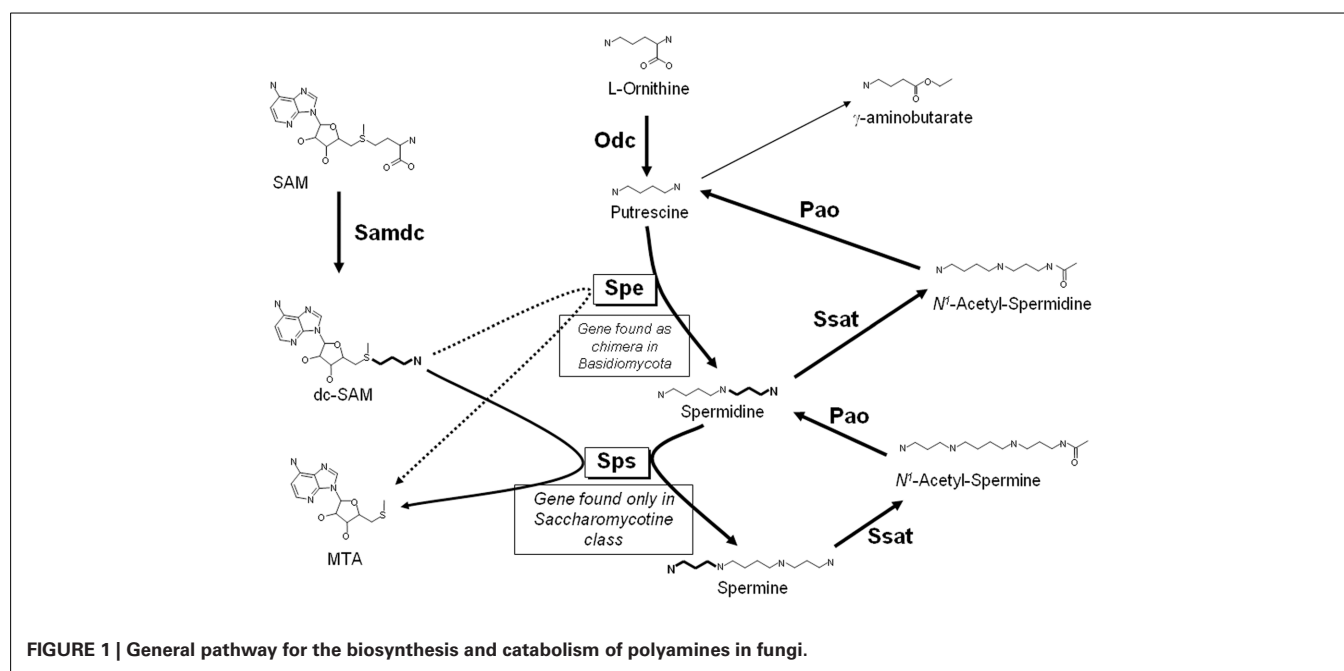
RETRO-CONVERSION OF POLYAMINES

In fungi, polyamines are oxidized to putrescine by the pathway shown in **Figure 1**. The first step is the acetylation of the aminopropyl group of polyamines, a reaction catalyzed by spermine or spermidine *N*¹-acetyltransferase (Ssat; E.C. 2.3.1.57), to give either *N*¹-acetyl-spermidine or *N*¹-acetyl-spermine. These are in turn degraded by a polyamine oxidase (Pao; E.C. 1.5.3.11), with the formation of either putrescine or spermidine. Polyamine acetyltransferases and polyamine oxidases have been reported to be present in yeast as well as in other fungi (Yamada et al., 1980; Chattopadhyay et al., 2003b; Landry and Sternglanz, 2003; Liu et al., 2005; Valdes-Santiago et al., 2010). The ability to direct back-conversion of spermine to spermidine by spermine oxidase such as occurs in animals has been reported

in plants and it is other dissimilarity between plants and fungi (Tavladoraki et al., 1998; Alcazar et al., 2006). In plants, redundancy of genes such as diamino oxidases or polyamine oxidases genes has been documented (Alcazar et al., 2006; Ono et al., 2012).

REGULATION OF POLYAMINE SYNTHESIS

Modulation of polyamine biosynthesis is mostly achieved by the degradation of Odc protein, as it has been reported in different species including *Schizosaccharomyces pombe*, *Neurospora crassa*, and *Saccharomyces cerevisiae* [(Barnett et al., 1988; Toth and Coffino, 1999) for review see (Ivanov et al., 2006)]. The regulator of Odc is the protein ornithine decarboxylase antizyme (Az) (Hayashi et al., 1996). Az interacts with Odc to be degraded by the proteasome in an ubiquitin independent manner (Zhang et al., 2003). Among fungi, the regulatory mechanism of Az is conserved [for review, see (Sorais et al., 2003)]. As indicated above, it was demonstrated that in *S. cerevisiae*, the degradation of Odc occurs without ubiquitination, as also happens in mammalian cells (Gandre and Kahana, 2002; Ivanov et al., 2006), and the identification of genes encoding Az from other fungi, suggests that the mechanism may be widely distributed in these organisms (Ivanov et al., 2006; Ivanov and Atkins, 2007). A peculiarity of fungi is that they present a single antizyme ortholog, while mammalian cells possess several antizyme encoding genes (Coffino, 2001a,b; Kurian et al., 2011). As expected, it is known that Az is positively regulated by polyamine levels. The mechanism for this regulation involves a translational frameshift in the open reading frame (ORF) in the encoding gene, through which a second ORF that encodes the active protein is established. In *S. cerevisiae* an unusual mechanism for the control of Az synthesis was unveiled (Kurian et al., 2011). Accordingly, the authors made the surprising discovery that at low polyamine levels Az acquires a



conformation that arrest its own synthesis, but high concentrations of polyamines bind, not to the regulatory region of the gene, but directly to the Az polypeptide, thus avoiding that it acquires such conformation, and promoting the completion of its synthesis. Other regulatory mechanisms of the metabolism of polyamines occur at the levels of Odc, Ssat, and Pao, due to their early and rapid responses to external stimuli (Vujcic et al., 2003; Wallace et al., 2003). Additionally, the existence of regulatory sequence elements in the 5' and 3' of *ODC* regions in *N. crassa* are related with changes in the rate of synthesis of Odc, and with changes in the abundance of *ODC* mRNA (Williams et al., 1992; Hoyt et al., 2000).

Contrary to fungi, plants possess several copies of the genes involved in polyamine metabolism which increase the complexity of polyamine regulation. As an example, in *Arabidopsis thaliana* there are two genes encoding *Adc1* and *Adc2*, the first one is presented in all tissues and it is constitutively expressed, while the second one responds to some abiotic stresses (Soyka and Heyer, 1999; Perez-Amador et al., 2002). Likewise, *Spd1* and *Spd2* are the genes encoding spermidine synthase in *A. thaliana* (Imai et al., 2004; Ge et al., 2006).

Regarding SAMDC, it is synthesized as a proenzyme, it has been demonstrated that putrescine induces the cleavage of the proenzyme in a specific amino acid to produce the active and mature enzyme (Pegg et al., 1998). In plants there is an additional regulation control at transcriptional level. Plant *Samdc* contain a tiny 5'-uORF and introns in 5' leader sequence that regulate their expression (Hu et al., 2005). The absence of Az homolog in plant genomes corroborates the predominance of SAMDC as the regulator of polyamine homeostasis (Illingworth and Michael, 2012).

INTERACTIONS WITH OTHER METABOLIC PATHWAYS

The pleiotropic effects observed in polyamine mutants may be due to the relationships existing between polyamines and other metabolic pathways. As an example, ornithine, the precursor of putrescine not only is considered a key regulator of polyamine biosynthesis, but it may also regulate the pathways for glutamate transformation to arginine and to proline. Indirectly, it can also regulate putrescine catabolism, contributing to the aminobutyric acid content of the cells, since putrescine can be converted into Δ^1 -pyrroline by an amino oxidase, and this compound is metabolized to γ -aminobutyrate by pyrroline dehydrogenase (Seiler et al., 1979; Fogel et al., 1981; Majumdar et al., 2013). Another compound related to polyamines is S-adenosylmethionine (SAM), which is essential for the synthesis of polyamines. S-adenosylmethionine synthetase (Sams; EC 2.5.1.6) catalyzes the biosynthesis of SAM from ATP and L-methionine (Tabor and Tabor, 1984). In *S. cerevisiae*, methionine regulates the expression of the two *SAMS* genes (*SAMS1* and *SAMS2*) (Thomas et al., 1988); and in turn *SAMS2* gene is subjected to the inositol-choline regulation given by the octameric sequence 5'-CATRTGAA-3' contained in its upstream promoter region (Kodaki et al., 2003). Genes encoding Sams are evolutionarily well conserved (Mautino et al., 1996). In *S. pombe* mutants, the absence of Sams affected cell growth, mating and sporulation, and by over-expressing

the gene, growth of the cells became methionine-sensitive (Hilti et al., 2000).

5'-Methylthioadenosine (MTA), is a product of SAM catabolism during polyamine biosynthesis (Heby, 1981). More than 98% of MTA in *S. cerevisiae* is a by product of SAM, originated from the polyamine biosynthetic pathway (Avila et al., 2004). MTA can affect gene expression, proliferation, differentiation and apoptosis, and these effects may be due to the inhibitory effect that intracellular accumulation of this nucleoside has over polyamine biosynthesis *in vivo* (Raina et al., 1982; Garcea et al., 1987); in addition in *S. cerevisiae*, MTA causes a specific inhibition of spermidine synthase (Chattopadhyay et al., 2006a).

Regarding plants, SAM is the precursor of ethylene, hence polyamines, DNA methylation and ethylene share it as a common precursor (Pandey et al., 2000). Polyamines are precursors of many plant secondary metabolites such as nicotine and tropane alkaloids (Martin-Tanguy, 2001). Furthermore, plant polyamine metabolism is also connected with the production of nitric oxide and GABA [Reviewed by Alcazar et al. (2010)]. The regulation of polyamine biosynthesis, proline and cytokinins by abscisic acid as well as ethylene during UV-B stress and salt stress has been documented (Rakitin et al., 2009; Xue et al., 2009; Shevyakova et al., 2013).

POLYAMINES AND STRESS

TOLERANCE OF FUNGAL MUTANTS AFFECTED IN POLYAMINE METABOLISM TO DIFFERENT STRESS CONDITIONS

The study of the stress response in fungal mutants affected in polyamine metabolism was started with *S. cerevisiae*, where the authors had in mind evidences indicating some roles of polyamines in the protection of the cell and cell components, and cell differentiation (Balasundaram et al., 1993; Chattopadhyay et al., 2006b; Watanabe et al., 2012). In general the ability to deal with stress is diminished in mutants affected in any of the genes encoding enzymes related with polyamine metabolism. *U. maydis* is a well studied system in this regard, proving to be an important model organism for understanding polyamine metabolism, especially since this phytopathogenic fungus lacks spermine (Valdes-Santiago et al., 2009).

In general, fungal spores are rather more resistant to different environmental stresses than vegetative cells. However, in the early steps of germination, when they lose their unique spore wall, they become more sensitive to different environmental stresses (Herman and Rine, 1997; Joseph-Strauss et al., 2007). Interestingly, it has been described that polyamines affect positively spore germination in *Glomus mosseae*, *Rhizopus stolonifer*, *Botryodiplodia theobromae*, *Gigaspora rosea*, and *Glomus etunicatum* (Nickerson et al., 1977; El Ghachtouli et al., 1996; Sannazzaro et al., 2004; Cheng et al., 2012). Inhibition of polyamine metabolism gave rise to an inhibition of spore germination and germ tube growth in fungi such as *Uromyces phaseoli* (Galston, 1989). Genes related with polyamine transport have been implicated in the germination process, and it was described that these genes are up-regulated compared to the vegetative state (Ruiz-Herrera, 1994; Dembek et al., 2013). These results suggest a relationship between polyamines and changes in the expression of

genes related to stress. During a study of *S. cerevisiae* sporulation-specific genes, two divergently transcribed genes *DIT1* and *DIT2*, were found to be repressed during vegetative growth via a common negative regulatory element, referred to as NER^{DIT} (Friesen et al., 1997). The authors reported that the spermidine synthase gene was required for complete repression through NER^{DIT} , and cells that could not synthesize spermidine not only failed to support complete repression, but also had modest defects in repression of other genes, suggesting that spermidine could modulate gene expression (Friesen et al., 1998). However, there is not yet a report about gene expression response of fungi to different environmental stresses that reveal information on what is the mechanism through which polyamines are operating in this context.

ROLE OF PHYSIOLOGICAL POLYAMINES ON OSMOTIC STRESS

In silico phylogenetic analyses have revealed that central components of the osmotic, oxidative and cell wall stress signaling pathways are relatively well conserved in fungi (Bahn et al., 2007; Nikolaou et al., 2009). Nevertheless, knowing that polyamines are important in the response to stress, and that their metabolic pathway has been conserved among all living organism; we may suggest that a principal role of polyamines is to promote the restoration of cellular homeostasis allowing survival under stressful conditions. In *S. cerevisiae*, the expression of the major permease for high affinity polyamine import coincided with the osmotic stress imposed by high concentration of NaCl, KCl, or sorbitol (Lee et al., 2002; Aouida et al., 2005). Also in yeast, it was observed that the serine/threonine protein kinases Ptk1p and Ptk2p were involved in the regulation of spermine uptake, and that disruption of *PTK2* gave rise to salt tolerance, while Ptk2p or Sky1p (another serine/threonine kinase that was found in spermine tolerant strains) over-expression, led to increased salt sensitivity (Erez and Kahana, 2001). These and other data clearly reveal that polyamines have a role in osmotic stress response, probably by regulation of the expression of osmotic stress signaling via protein kinases (Auvinen et al., 1992; Shore et al., 1997; Flamigni et al., 1999).

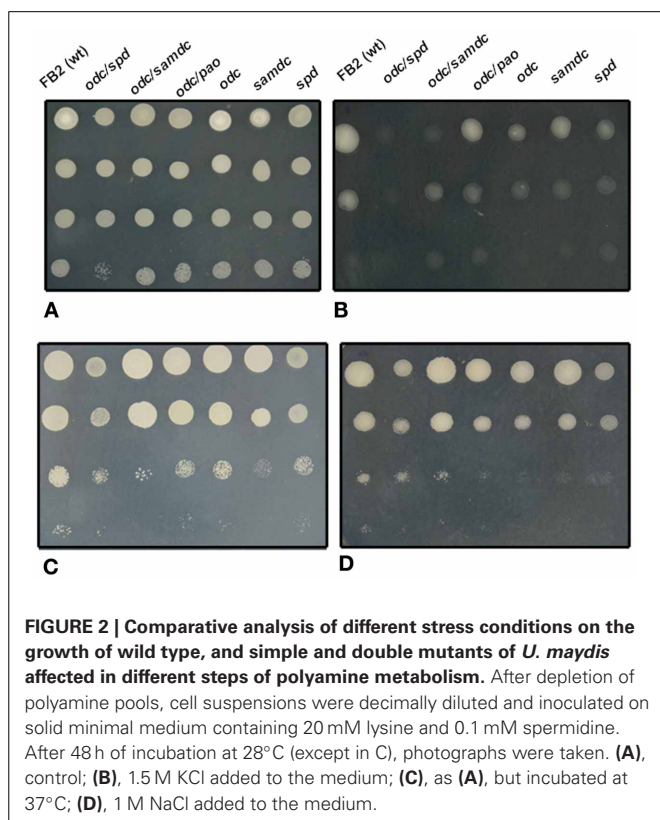
As would be expected, it is known that the lipid composition of the membrane affects the osmotic stability of the yeast plasma membrane (Allakhverdiev et al., 1999). In consequence, it was important to establish if the sensitivity of polyamine mutants to different kinds of stress was due to a defect at the membrane level or to an indirect alteration of other signaling pathways controlled by polyamines. In this sense, data exist showing that osmotic shock in different systems affects the intracellular levels of polyamines. For example, it was reported that under osmotic shock the content of putrescine was increased up to 60-fold in oat, barley, corn, wheat, and wild oat leaves (Flores and Galston, 1982); and the induction of Odc activity under hypo-osmotic stress leading to an increase in the polyamine pool was observed in L1210 mouse leukemia cells (Poulin et al., 1991). The effect of a high osmotic concentration was tested in *U. maydis* mutants affected in the gene encoding spermidine synthase (*spd*), and in double mutants affected also in the ornithine decarboxylase gene (*odc/spd*). Both simple and double mutants showed no significant differences in their growth rate in the absence of salt stress,

when compared to the wild type, but growth rate of the same cells treated with 1 M KCl or 0.3 mM SDS was severely inhibited in comparison with the wild type strain. This result demonstrates a role of spermidine in protecting the fungus from different deleterious factors that affect the cell membrane (Valdes-Santiago et al., 2009). When mutants affected in the polyamine oxidase gene (*pao*), and double mutants affected in ornithine decarboxylase and polyamine oxidase (*odc/pao*) were subjected to ionic or osmotic stress (1 M NaCl, 10 mM LiCl, or 1 M sorbitol), only the double mutant displayed a sensitive phenotype, whereas the simple *pao* mutant showed no differences to the wild type strain (Valdes-Santiago et al., 2010).

To understand the effects of polyamine deficiency on the phenotypic alterations suffered by the cell subjected to osmotic stress, it is important to take into consideration the existence of specific mechanisms involved in cation handling, and the possible effect of some pathways that interact with polyamine metabolism. This fact was clearly demonstrated by the comparison of the behavior of *U. maydis* S-adenosylmethionine decarboxylase (*samdc*) and *spd* mutants under stress conditions. Growth rate of both mutants was substantially inhibited by 1 M NaCl, but only the *samdc* mutant was affected by 10 mM LiCl. A fundamental difference between *samdc* and *spd* mutant is that, unlike the wild-type strain, they accumulate high levels of SAM, but *spd* mutants also accumulate SAM. The other metabolite involved in the pathway is dcSAM, which was 46-fold increased in the *spd* mutants (Valdés-Santiago et al., 2012b). The authors explained these differences in the phenotype in relation to their different levels of SAM and dcSAM, which could affect DNA methylation, and other different cellular functions, plus the possibility that sodium and lithium are managed by different transporters.

A further example of the specificity of polyamines in osmotic stress was the observation that in *Synechocystis* sp., a cyanobacterium, salt stress induced an increase in the spermine content, whereas an osmotic stress induced a moderate increase in the total spermidine content (Jantaro et al., 2003). Interestingly, this response was correlated with an increase of arginine decarboxylase mRNA levels, and an increase in the uptake of putrescine and spermidine (Incharoensakdi et al., 2010). In this sense, we may cite that simple or double *U. maydis* polyamine mutants presented a variety of stress sensitivities to osmotic stress (**Figures 2B,D**), compared with wild type and same mutants without stressful agent (**Figure 2A**).

In plants it has been well established that general polyamine biosynthesis is modified under salt stress at transcriptional level [for review see (Mutlu and Bozcuk, 2007; Liu et al., 2008; Alcazar et al., 2010; Gupta et al., 2013)]. However, spermine seems to have a specific role considering that, this is the polyamine reported to be more affected regardless of species, varieties and plant tissue studied. To mention some examples, plants such as *A. thaliana* unable to produce spermine showed hypersensitivity to high levels of NaCl and KCl and the mechanism behind polyamines protection of salt stress has been studied. Some reports suggest that, polyamines improve ionic equilibrium by modifying the plasma membrane to overcome the osmotic stress since the expression of some genes belonging to signaling pathway related with salt stress were not altered in spermine deficient



mutant. Moreover, spermine seemed to modulate Ca^{2+} permeable channels and change Ca^{2+} allocation restraining the entry of Na^+/K^+ to the cytoplasm (Yamaguchi et al., 2006; Janicka-Russak et al., 2010; Najmeh et al., 2012; Velarde-Buendia et al., 2012). Other reports described that spermidine or putrescine are the polyamines conferring protection under salt stress, although it is difficult to support this conclusion, since putrescine or spermidine can be inter-converted (Quinet et al., 2010; Saleethong et al., 2011). In this respect, mutants are very effective in avoid confused results since they can define whether polyamines are produced by *de novo* synthesis or back-conversion (Valdes-Santiago et al., 2010).

OXIDATIVE STRESS RESPONSE

Fungi, as well as other microorganisms, must deal with the danger of oxidative stress under different scenarios. An important example is the oxidative killing of fungal cells by the host defense mechanisms, and sometimes the ability to proliferate in the host has been correlated with the expression of redox active enzymes such as catalase (Wysong et al., 1998; Moye-Rowley, 2003). Accordingly, it has been concluded that one of the polyamine functions is the cell protection from damage caused by ROS (Rider et al., 2007; Cerrada-Gimenez et al., 2011). In the case of *E. coli*, oxidative stress induces the expression of catalase, hydroperoxide reductase and glutathione reductase (Storz and Imlay, 1999; Chattopadhyay et al., 2003a), and interestingly, putrescine is up-regulated the bacterium in a concentration-dependent manner with the expression level of the transcription

factor controlling the genes already mentioned (Kim and Oh, 2000; Tkachenko et al., 2001). In the case of *U. maydis* it was observed that *pao* and *odc* polyamine mutants grown on agar plates where 0.8 mM H_2O_2 -containing paper disks were placed, showed halos of inhibition wider in comparison with the wild-type strain; i.e., they were more sensitive than the wild type to the oxidative stress (Valdes-Santiago et al., 2010).

In fungi in general the mechanism regulating their response to oxidative stress has been described to involve the control of oxidant-responsive factors, such as Yap1p at level of cell localization and by regulation of enzyme activity via protein phosphorylation (Moye-Rowley, 2003). When *S. cerevisiae* was affected in the production of spermidine and spermine because of a deletion of the *Samdc* gene; there occurred a loss in cell viability when cultures were incubated under an oxygen atmosphere (Balasundaram et al., 1993).

It has also been shown that yeasts affected in the production of spermidine accumulate ROS, and show an increased sensitive to oxidative damage. In this regard, polyamine-deficiency in *S. cerevisiae* induced accumulation of ROS that led to the development of an apoptotic phenotype (Chattopadhyay et al., 2006b). These authors showed that one of the polyamine functions was the protection to the accumulation of ROS. There is yet to know whether polyamines act by affecting the enzymes involved in the synthesis or degradation of ROS, or by a direct interaction with ROS. Some evidences point out to some hypothetical polyamine-binding sites in proteins involved in these different processes (Watanabe et al., 2012). In this same respect, it has been proposed that spermine acts by direct scavenging of reactive agents (Ha et al., 1998; Fujisawa and Kadoma, 2005), whereas other authors have suggested an inhibition of the activity of NADPH oxidase (Papadakis and Roubelakis-Angelakis, 2005), and a role of polyamines as regulators of the MAPK signaling pathway (Stark et al., 2011).

In plants, a swift accumulation of ROS is presented under stressful conditions (Gill and Tuteja, 2010b; Suzuki et al., 2012); and it has been observed that intracellular ROS produced by polyamine catabolism are essential during development. Thus, it has been described that H_2O_2 generated during their oxidation and back-conversion serve as a signaling molecule correlated with plant defense and stress response (Moschou and Roubelakis-Angelakis, 2011; Pottosin et al., 2012). Accordingly, in *Salvinia natans* response to salinity there occurs an interaction between ROS formation, and the expression of *Adc*, *Samdc*, *Spd*, and *Spm* as well as *Pao* (Tanou et al., 2013).

TEMPERATURE STRESS

Temperature has critical effects on microbial metabolism and cellular composition (Bennett et al., 1992; Feder and Hofmann, 1999; Fargues and Luz, 2000; Gavito and Azcon-Aguilar, 2012). It has been suggested that polyamines are involved in the stabilization of cellular components at high temperatures; for example a *Tapesia yallundae* *odc* null mutant exhibited temperature-dependent growth: at high temperatures, hyphal elongation was more restricted in comparison to the wild type, and the hyphae were thinner, less melanized, and grew sparsely (Mueller et al., 2001). Similarly, an *S. cerevisiae* mutant unable

to synthesize spermidine or spermine, because it was affected in the gene encoding S-adenosylmethionine decarboxylase, was more sensitive to elevated temperatures than the parental strain (Balasundaram et al., 1996). The opposite takes place when polyamines are accumulated. Under these conditions, the cells present resistance to high temperature stress (Cheng et al., 2009). Simple and double *U. maydis* mutants affected in polyamine metabolism also presented a temperature sensitive phenotype in agreement with the reports above mentioned (see **Figure 2C**).

In plants, polyamine alterations have been correlated with temperature changes; under cold treatment putrescine levels were increased together with the expression of *Adc1*, *Adc2*, and *Samdc2* in *A. thaliana* (Urano et al., 2003; Cuevas et al., 2008), while the addition of putrescine had a positive effect over cotton seed subjected to high temperature (Bibi et al., 2010). These results are in agreement with reports on *Arabidopsis thaliana*, where a mutant affected in the gene encoding spermine synthase was hypersensitive to heat shock; whereas an overexpression of spermine synthase-encoding gene conferred thermotolerance to the cell (Sagor et al., 2013). In the same study it was established a direct correlation between spermine content and the expression of heat shock genes and proteins suggesting that there is a control of polyamines at transcriptional and translational levels to induce the protection of plants under temperature stress. It is interesting to notice that although *U. maydis* does not contain spermine, spermidine covers the function to resist temperature stress (**Figure 2C**).

In the same line, it has been suggested that the mechanism behind the sensitivity of polyamine-deficient cells could be related to the stability that polyamines may provide to some cell components (Schuber, 1989).

POLYAMINES IN STRESS PRODUCED BY PATHOGEN-HOST INTERACTIONS

It is well known that during the course of host colonization, fungal pathogens need to overcome a wide range of challenges such as oxidative burst, which results in the production and accumulation of ROS. It has been proposed possible roles for polyamines and polyamine catabolism in plant resistance to pathogen infection (Walters, 2003). In this context, plants would activate polyamine oxidation to produce hydrogen peroxide, which would lead plant defense mechanisms. Yoda et al. (2003) confirmed these data, when they correlated hypersensitive response with the accumulation of polyamines in the apoplast of *Arabidopsis thaliana* infected with *Pseudomonas syringae*, and of rice infected with *Magnaporthe grisea* (Yoda et al., 2003). Expression of *Odc*, *Adc*, and *Samdc* genes in *Theobroma cacao* were induced under different stresses such as, drought and infection with *Phytophthora megakarya*, or the necrosis inducing protein Nep1 from *Fusarium oxysporum* while *Spds* and *Sps* were not changed (Bae et al., 2008). In the same manner, H₂O₂ produced by Paos during hypersensitive response provoke disease tolerance against *Pseudomonas syringae* pv tabaci and *Phytophthora parasitica* var nicotianae (Yoda et al., 2003; Moschou et al., 2009).

Spermine has been proposed as the polyamine that contribute to defense signaling against plant pathogens through the regulation of defense-related genes. In this sense, an *A. thaliana* mutant

that overexpressed *Sps* gene, displayed higher resistance to infection with *Pseudomonas viridiflava*, whereas a mutant with low spermine levels showed hyper sensitivity; interestingly the overexpression of *Sps* was accompanied by up-regulation of genes involved in disease resistance protein, as well as several transcription factors (Cona et al., 2006; Kusano et al., 2008; Gonzalez et al., 2011). Likewise, spermine-responsive genes were detected in *A. thaliana* during infection with cucumber mosaic virus, and interestingly, blocking of spermine oxidation abolished induction of these genes (Mitsuya et al., 2009). In general, response from pathogenic organism with distinct strategies to cause disease either necrotrophic or biotrophic, is essentially analogous, as was demonstrated with *Sclerotinia sclerotiorum* and compared with some *Pseudomonas species* or *U. maydis* (Marina et al., 2008; Rodriguez-Kessler et al., 2008; Rodriguez-Kessler and Jimenez-Bremont, 2009). In these two cases what was observed was the induction of polyamines connected with the activation of genes encoding polyamine biosynthesis enzymes. Nevertheless, it is important to mention that changes in plant polyamine metabolism by transgenic strategies allow the modification of plant responses to pathogenic organisms. The expression of yeast *Spe* gene and accumulation of polyamine levels in tomato led to an increasing of sensitivity to the attack by *Botrytis cinerea*, but not by *Alternaria solani* and the normal response to the attack was restored by polyamine inhibitors (Marina et al., 2008; Nambeesan et al., 2012).

From the side of the pathogen, the state of polyamine metabolism affects their interaction with the host. When this *Streptococcus pneumoniae* was affected in polyamine transport, the mutant strain showed attenuation in virulence in a murine model. And it has been reported that human bacterial pathogens use different strategies related with polyamines to infect their hosts (Ware et al., 2006; Di Martino et al., 2013). A polyamine mutant of *Salmonella enterica* serovar typhimurium, was unable to invade and survive intracellularly in *Caenorhabditis elegans*, and showed no systemic infection in a mouse model of typhoid fever (Jelsbak et al., 2012). *Francisella tularensis*, *Yersinia pestis* and *Vibrio cholerae* are other systems in which synthesis and transport of polyamines were found to be important for virulence (Wortham et al., 2010; Russo et al., 2011; Goforth et al., 2013). Regarding fungi, a *spe* mutant of the thermally dimorphic fungus *Penicillium marneffe*, a pathogen of immune compromised persons, showed defects in pathogenesis, conidiogenesis, spore germination, and growth. These results led to suggest that the spermidine biosynthetic may serve as a potential target for combating infections (Kummasook et al., 2013). Agreeing with these ideas, in another pathosystem reviewed by Valdés-Santiago et al. (2012b): *U. maydis*-maize, it was observed that *samdc* mutants of the fungus were avirulent.

GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

Along this article, evidences indicate multiple roles of polyamines in cell survival during stress. A strategy toward the knowing of the mechanism behind polyamines action should include firstly the clear identification of the specific roles of each polyamine i. e., in *U. maydis* putrescine might be controlling stress response since the mutant (*odc/pao*) was over sensitive to stress, in comparison

to the wild type and and *pao* mutant. Also, spermidine appeared to control differentiation of this fungus, since mutant it was able to carry out a dimorphic transition only when supplied with high concentration of spermidine (Valdes-Santiago et al., 2010). Once the specific roles of each polyamine are known, it will be possible to distinguish the role of other polyamines in the general aspect of cell physiology. This review clearly stresses the fact that changes in polyamine metabolism affect the response of fungi to different types of stress. This fact by itself is an evidence of the importance of polyamines in cell survival.

Although a large advance in our understanding on polyamine metabolism in fungi has taken place in recent years, some aspects are still poorly understood, especially regarding polyamine transport, distribution in the cell, and regulation of metabolism. It is also clear that the role of polyamines in stress-response mechanisms in fungi, and their mode of action have been insufficiently analyzed. We consider that it is necessary to have a global view of the physiology of stress response in fungi that includes polyamines as an important player, possibly using mutants affected in different steps of polyamine metabolism and its regulation, and studies of the relationships of polyamine metabolism with other important metabolic and regulatory processes of the cell. If this expectancy is fulfilled, undoubtedly that fungi will become important models that could help to unravel the mechanism of the protection exerted by polyamines to stress in general.

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Aptamer based electrochemical sensors for emerging environmental pollutants

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Environmental contaminants monitoring is one of the key issues in understanding and managing hazards to human health and ecosystems. In this context, aptamer based electrochemical sensors have achieved intense significance because of their capability to resolve a potentially large number of problems and challenges in environmental contamination. An aptasensor is a compact analytical device incorporating an aptamer (oligonucleotide) as the sensing element either integrated within or intimately associated with a physiochemical transducer surface. Nucleic acid is well known for the function of carrying and passing genetic information, however, it has found a key role in analytical monitoring during recent years. Aptamer based sensors represent a novelty in environmental analytical science and there are great expectations for their promising performance as alternative to conventional analytical tools. This review paper focuses on the recent advances in the development of aptamer based electrochemical sensors for environmental applications with special emphasis on emerging pollutants.

Keywords: electrochemical sensor, aptamer, emerging pollutants, environmental applications, analytical monitoring

INTRODUCTION

Nucleic acid, as the biological polymer for the storage and propagation of genetic information, possess remarkable structural and functional characteristics (Tang et al., 2014). Highly specific base pairing properties of nucleic acid permit for replication and transcription processes (Seeman, 2003). Recently, nucleic acid has been emerged as a powerful and versatile building block in the construction of biosensors (Seeman, 2010). A variety of nucleic acid (DNA or RNA) based biosensors have been designed and reported in the literature. Among them, aptamers are nucleic acid (DNA or RNA) that selectively bind to low molecular weight organic or inorganic substrate or to relatively big macromolecules. The affinity constant of aptamers toward their specific targets is reported in the micromolar to pico molar ranges, comparable to the binding constant of antibody and antigen affinity interaction (Jenison et al., 1994; Willner and Zayats, 2007). They are engineered through an *in vitro* selection procedure, also called SELEX (Systematic Evolution of Ligands by EXponential enrichment), which was first reported in 1990 (Hayat et al., 2012b, 2013c; Paniel et al., 2013).

Recently, aptamers have found tremendous interest as an active separation material in chromatography and electrophoresis, therapeutic and diagnostic agent, and as acting recognition material in biosensing to replace the commonly used bioreceptors (Ellington and Szostak, 1990; Hermann and Patel, 2000; Hamula et al., 2006; Tan et al., 2013). Aptamers offer many advantages compared to antibodies, which are biologically produced antigen specific proteins. The production of aptamer does not require an immune response in host animals to obtain them, as they are chemically produced by automated nucleic acid

synthesis. Similarly, the antibodies cannot be easily obtained for small size targets (e.g., metal ions) or for molecules with poor immunogenicity or high toxicity, while there is a possibility to design aptamer against such target analytes. Besides, aptamers can be very easily chemically modified which permits to immobilize them over wide range of transducer surfaces (O'Sullivan, 2002; Gorodetsky et al., 2008). Moreover, the properties of conformational changes upon target-analyte binding make them most appropriate and suitable candidate to design label free and portable biodevices for analytical applications. This conformational alteration characteristic of aptamer facilitates and enhances the detection phenomena of small size target analytes by enfold-ing them in the folded DNA structures. For large molecules such as proteins, the folded DNA aptamer bind to a particular epitope. In principle, aptamer based biosensors can be fabricated to respond to any ligand for which an aptamer exists (Wang et al., 2011; Tang et al., 2014). They are widely regarded as ideal recognition element for diverse analytical applications, particularly environmental analysis.

Recent years have witnessed increasing need to monitor the environmental contaminations. Food, air and water are the main victims of the contaminants that may have impact on human and animal life. The environmental contaminants have mild to severe short-term or long term effect and some of them even have deadly effects and lead to widespread havoc. The contaminants that need monitoring in the environment can be broadly classified as small organic and inorganic pollutants, pharmaceutical and personal care products, toxins of microbial origin and pathogens. Although there has been lot of interest in developing techniques for monitoring of environmental pollutants, there is

still great demand for portable, decentralized and highly robust assays (Cella et al., 2011). Chromatographic methods are the traditionally used assays for quantitative and qualitative measurement of environmental pollutants. Although these methods are very sensitive and selective, but they still require costly instruments and trained person to perform the analysis, in addition to being unsuitable for decentralized analysis. Biosensors based on the antibody as bio-recognition element have been emerged for environmental monitoring. Because of the expensive animal models required to produce antibody, unavailability against non-immunogenic contaminants and instability under varying physiological conditions, antibodies are not potential candidates for environmental monitoring analysis. Alternatively, RNA or DNA Aptamers have attained great attraction in the field of environmental monitoring. Apart from having the same or even higher sensitivity and selectivity as antibodies, aptamers offer the advantages of large scale production with less expensive *in vitro* system and enhanced environmental stability. Aptamers due to their ease of modification with various functional groups can be integrated into electrochemical biosensing platform. This review summarizes the accomplishment, and highlights the advantages of electrochemical aptasensors for environmental samples analysis.

ELECTROCHEMICAL SIGNALING OF APTAMER CONSTRUCTS

Transduction of the affinity binding event to measurable signal is usually obtained through optical output in aptamer based assays. Traditionally optical based read out methods of aptamer binding event not only require high precise and expensive instrumentation but also involve sophisticated numerical algorithms to interpret the data. Alternatively, a number of innovative designs of electrochemical aptasensors have been reported in the literature. This type of devices combined aptamer with electrochemical transducers to generate an electrical signal, and provides a simple, accurate and an inexpensive platform for applications such as environmental monitoring.

ADVANTAGES OF ELECTROCHEMICAL METHODS

Among all the transduction approaches, electrochemical detection is an attractive sensing platform in the field of biosensors (Barthelmebs et al., 2011; Hayat et al., 2011, 2012a). It was not explored in aptasensing until 7 years ago; however, since these last years, electrochemical transduction has received much attention to monitor the behavior of target-aptamer complex on electrode surface (Hayat et al., 2013a,b). An electrochemical device measures change in the electric current produced by redox reactions occurring on the transducer electrode surface (Hayat and Andreescu, 2013). This sensing methodology has various advantages including simplicity, rapidity, low cost and high sensitivity. The use of aptamer as bio-recognition element is strongly favored for some electrochemical detection techniques. Till now, plenty of research work has been devoted to the development of electrochemical aptasensors. Electrochemical aptasensor for biomolecular reaction monitoring is mainly based on the detection of current or potential changes resulting from interactions occurring at the transducer surface. It presents several advantages over optical, piezoelectric or thermal detection. Electrochemical transduction is simple, rapid, cost effective, highly sensitive and

selective, compatible with novel micro-fabrication techniques, disposable, easy to miniaturize, robust, and independent of sample turbidity, in view of high-throughput. Electrochemical reactions usually provide an electronic signal directly, avoiding the requirement of expensive signal transduction equipment (Castillo et al., 2012; Rhouati et al., 2013a,b).

As aptamer are nucleic acid strand and can be very easily immobilized on the transducer electrode surface, chip-based, portable, and miniaturized electrochemical systems can be designed accordingly. For electrochemical aptasensor, most of the commonly used electrode materials are gold and carbon surfaces on which aptasensor construct can be built via different immobilization strategies and detection formats (Figure 1) (Lai et al., 2006; White et al., 2008).

CONVENTIONAL METHODS FOR ELECTROCHEMICAL APTASENSING

There is a great variety of different labels which have been applied in electrochemical aptasensors. These reporters can be covalently conjugated to the aptamer itself, conjugated to a complementary oligonucleotide, or indirectly attached with aptamers. Following the incorporation of an electrochemical reporter, detection can be either signal-on (Figure 2) or signal-off (Figure 3), depending on the format of the assay. Among the most valuable labels are enzymes such as horse radish peroxidase (HRP), glucose oxidase (GOD), alkaline phosphatase (ALP). Other reporters can also be used to overcome problems typically associated with enzymes. In particular, electroactive compounds such as ferrocene, ferrocyanide, methylene blue (MB), Pt and Cds quantum dots (QDs), and other nanoparticles (NPs) offer a number of advantages over standard enzymes for monitoring biological systems (Ikebukuro et al., 2005; Mir et al., 2006; Centi et al., 2007; Park et al., 2011).

REDOX LABELING METHODS FOR APTASENSORS

Sensitive electrochemical signaling is usually based on the redox properties of the reported molecules confined to the transducer surface. Different redox labels for electrochemical aptasensor have been developed in the past two decade. Various reporter

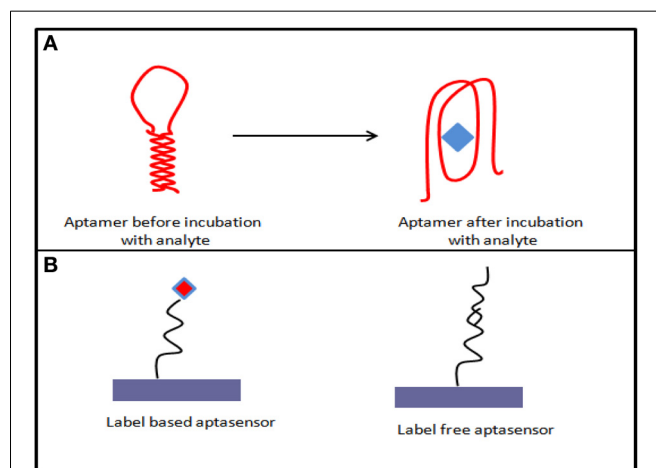
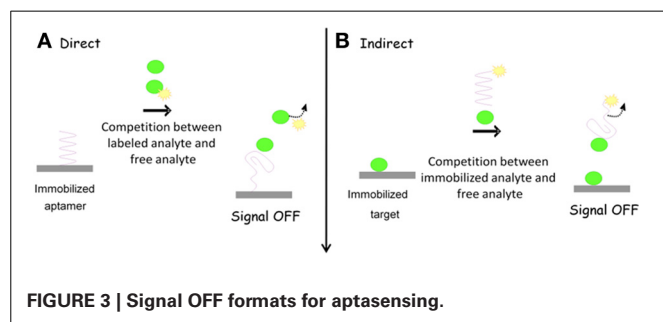
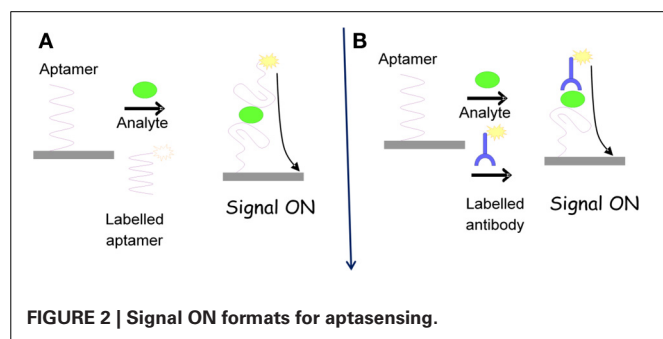


FIGURE 1 | (A) Aptamer conformational changes after target analyte incubation; **(B)** various possible formats of aptasensing.



molecules including ferrocene and methylene blue have been covalently tethered to the distal terminus of aptamer through flexible alkyl linkages. Based on the restriction of redox reporter mobility, the position of the aptamer can be controlled by varying their orientation or surface density. When the length of alkyl linkages is limited, the reaction is only a surface controlled process, without involvement of diffusion processes resulting in simplified electrochemical output data (Ihara et al., 1996; Pheeney and Barton, 2012). Intercalation is another procedure to insert a ligand between the base pair of DNA through non covalent interactions. The ligands called intercalators mostly include polycyclic, aromatic, and planar molecule (Kelley et al., 1997, 1999). Similarly, negatively charged reports which are freely diffusing in solution, cannot get into close proximity with the aptamer modified transducer surface due to electrostatic interactions. For example ferri/ferrocyanide ions in the solution are repelled by the negatively charged species, increasing the electron transfer resistance. This increase in electron transfer resistance can be measured as a function of analyte concentration by using electrochemical impedance spectroscopy (Yu et al., 2003; Cheng et al., 2007).

RATIONAL DESIGN OF ELECTROCHEMICAL APTASENSORS

In functional aptamer based electrochemical sensors, aptamer undergoes various structural or conformational changes in the presence of external stimuli, resulting in an alteration in the electrochemical signal. The most commonly explored pattern in this context is aptamer-ligand binding phenomena. When aptamers are immobilized on the electrode surface, the binding of the target analyte may induce structural and conformational changes. In the presence of redox reported, electrochemical signals can be obtained by employing conventional electrochemical techniques. Aptamer constructs are mainly single or double stranded in which

the target binding induces the dissociation of duplexes or the folding of single strands. Aptamers may bind to their target analytes by two different recognition events; (a) binding to their ligands and (b) binding to their Watson-Crick complementary strand. This competition can be employed to design novel types of electrochemical aptasensors. These types of formats form a fully or partially double stranded DNA sensor with one strand incorporating aptamer sequence. Upon incubation with target analyte, the aptamer selectively binds to the analyte with the dissociation of the DNA duplex, which is further employed to get the electrochemical signal (Zuo et al., 2007; Lu et al., 2008; Chakraborty et al., 2009). Alternatively, folding of single strand aptamer is used to construct the electrochemical sensors. This type of aptasensor design offer advantages such as; (a) the aptamer conformational changes upon target analyte bindings are simple and does not involve the dissociation of the duplex, (b) surface can be very easily regenerated, without requirement of re-hybridization of the dissociated strand. The later type of format is mostly commonly used to fabricate simple and robust electrochemical aptasensors (Xiao et al., 2005; Baker et al., 2006; Radi et al., 2006).

DIFFERENT METHODS OF APTAMER IMMOBILIZATION

Immobilization of aptamer on the transducer surface is one of the fundamental and critical steps in the design of electrochemical aptasensors. Aptamer can be immobilized on electrode surface via 5'-end or the 3'-end. However, the 3' end is more suitable because it simultaneously confers resistance to nuclease after its binding to electrode surface. Physical adsorption method by means of electrostatic forces is the simplest strategy to immobilize the aptamer on the transducer surface. Aptamer immobilization methods generally operate through chemisorption of thiol onto gold, followed by formation of self assembled monolayer (SAMs) by the attachment of amine-terminated aptamer to a thiol end group. Similarly, thiol-tethered aptamers have been reported for planar gold (Baker et al., 2006; Li et al., 2008). This method, however, has the disadvantage of low stability caused by desorption from the surface. The use of gold nanoparticles (NPs) as immobilization support for aptamer to fabricate aptasensors has also been very well established. The immobilization of aptamer on gold NPs has been performed through direct attachment on gold using a symmetric mixed disulfide (Liu and Lu, 2004). The other used format is a complex dipstick in which two different types of gold NPs are used. Metal NPs have also been used for the immobilization of DNA and could be a useful alternative to gold NPs in future (Wu et al., 2007).

To perform covalent attachment to chemically modified surface, aptamers labeled with chemical functional groups interact with the corresponding chemical groups to form a layer of ordered film of aptamer on the sensor surface (Lee et al., 2009; Actis et al., 2011). The most commonly used groups for surface attachment are hydroxyl, amine, and carboxylic acid surface functional groups. Silicates and silicones have also been used as immobilization support for the fabrication of aptasensors (Zhu et al., 2006). The interaction between avidin (or streptavidin) and biotin has been exploited for surface immobilization of number of biorecognition elements, including aptamer (Becker and Wilchek, 1972). Avidin or streptavidin can easily be immobilized on the

electrode surface either through physical adsorption or chemical bonding. The immobilization of aptamers on the transducer surface by hybridization with partially complementary oligonucleotide is another fixation approach. Oligonucleotides complementary to aptamers are immobilized on the electrode surfaces. However, due to the involvement of annealing and hybridization, it is difficult to control the experimental conditions (Zhang et al., 2007; Han et al., 2009). Electrochemical aptasensors have also been fabricated by incorporating aptamers into single walled carbon nanotubes (So et al., 2005). Similarly, multiwalled carbon nanotubes have been used as modifier to immobilize amine linked aptamer on the screen printed carbon electrodes.

ELECTROCHEMICAL APTASENSOR FOR ENVIRONMENTAL MONITORING

Environment can be contaminated by several chemical compounds, toxins, pathogens... etc. resulting in different types of environmental borne diseases. As an example, according to World Health Organization (WHO), food borne and waterborne diarrheal diseases results in 2.2 million deaths per annum, and out of which 1.9 million are children (<http://www.who.int/foodsafety/en/>). The increasing public health problems and concerns and subsequently their consequences demands the design and fabrication of more reliable and field suitable technologies for on site and cost effective monitoring of environmental contaminants (Sett et al., 2012). In this context, electrochemical aptasensors have been emerged as potential candidate in monitoring environmental contaminants (Figure 4, Table 1). Electrochemical aptasensor have the potential to address many of the challenges associated with the traditional methods with similar or improved

specificity and affinity characteristics in comparison to antibody based assays/sensors. Based on the nature of the target analyte, electrochemical aptasensor designed for environmental monitoring are classified into two major groups, for simplicity and better understanding of the scientific community.

1. Electrochemical aptasensor for small molecules detection.
2. Electrochemical aptasensor for pathogen detection.

ELECTROCHEMICAL APTASENSORS AGAINST SMALL MOLECULES

Small target analytes including antibiotics, toxins, pesticides and heavy metals can be present in a variety of environmental samples. One such example is theophylline, commonly used bronchodilator used for asthma patients, however, its overdose lead to sever toxicity like seizure.. etc. For this molecule, RNA based electrochemical aptasensors have been reported in the literature to monitor its level in human serum (Ferapontova et al., 2008). Similarly, aptamers against drugs such as cocaine have been developed and successfully implemented for their detection (Chen et al., 2008).

Electrochemical aptasensors against antibiotics

Antibiotics are used to farm animal along with their feed for prophylactic and therapeutic purpose. However, a certain amount of these antibiotics remains un metabolized and accumulate in the tissue or excrete in the surroundings (Chen et al., 2008). The presence of antibiotics in the environment may results in antibiotic resistance with the subsequent possibility of transmittance to the human being through food chain. Chloramphenicol, antimicrobial drug, has lost its favor due to resistance and serious side effects like aplastic anemia. Burk et al. were the first one who designed and reported an aptamer against chloramphenicol (Burke et al., 1997). However, RNA aptamers are suspected to nuclease attack and needs transcription and reverse transcription, making it difficult to screen. Recently, Metha et al. developed DNA aptamers, and the designed aptamers were used for the electrochemical detection of chloramphenicol. The aptamers were immobilized onto gold electrode surface via self-assembly approach. The developed aptasensors were very sensitive and selective toward detection of chloramphenicol (Mehta et al., 2011). Tetracyclines are another group of broad spectrum antibiotics which inhibits prokaryotic translation (Spahn and Prescott, 1996). They are often used as veterinary drug to promote growth in animals. Their residues have been detected in meat, milk, honey, eggs etc. (Pena et al., 1999; Muriuki et al., 2001). On the other hand, this antibiotic has also been reported as hepatotoxic to pregnant women (Gwee, 1982). Traditional detection methods such as HPLC have been failed for their detection due to lack of specificity. Recently, aptamer have been selected for the detection of tetracyclines (Berens et al., 2001). In this context, Kim et al. recently designed an electrochemical aptasensor over glassy carbon electrodes (Kim et al., 2010). After this work, many electrochemical aptasensors were designed and reported in the literature for the detection of tetracyclines (Kim et al., 2010; Zhang et al., 2010; Jeong and Rhee Paeng, 2012). Xiao et al. improved the structure of the aptamer to obtain a very high affinity constant against tetracyclines ($K_d \sim 0.8 \text{ nM}$) (Xiao et al., 2008). Zhang

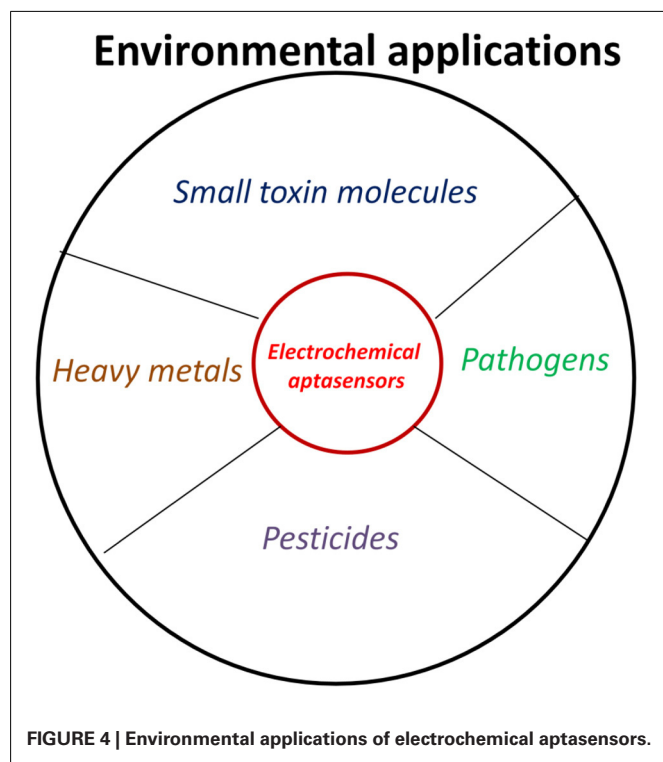


FIGURE 4 | Environmental applications of electrochemical aptasensors.

Table 1 | Electrochemical aptasensors designed for the detection of different target analytes.

Sr no	Analyte	Transduction methodology	Year	References
1	Theophylline	RNA aptamer-based biosensor	2008	Ferapontova et al., 2008
2	Cocaine	Aptameric biosensor	2008	Chen et al., 2008
3	Chloramphenicol	RNA aptamers	1997	Burke et al., 1997
4	Chloramphenicol	DNA aptamers	2011	Mehta et al., 2011
5	Tetracycline	RNA aptamer	2001	Berens et al., 2001
6	Tetracycline	Electrochemical aptasensor	2010	Kim et al., 2010
7	Tetracycline	Aptamer biosensor	2010	Zhang et al., 2010
8	Tetracycline	Aptamer-based assay	2012	Jeong and Rhee Paeng, 2012
9	Mycotoxins	DNA aptamer	2008	Cruz-Aguado and Penner, 2008
10	Endotoxin	Electrochemical aptasensor	2012	Kim et al., 2012
11	Bisphenol a	DNA aptamers	2011	Jo et al., 2011
12	Acetamidiprid	DNA aptamer	2011	He et al., 2011
13	Pesticides	DNA aptamers	2012	Wang et al., 2012
14	Virus-infected cells	Aptamers	2009	Tang et al., 2009
15	<i>Escherichia coli</i>	Aptamer-functionalized carbon-nanotube	2008	So et al., 2008
16	Anthrax	Nano aptasensor	2010	Cella et al., 2010

et al. reported a rapid electrochemical aptasensor using aptamers immobilized over glassy carbon electrodes. The aptasensor was able to rapidly detect tetracycline in milk with very high sensitivity (Zhang et al., 2010). Similarly, a competitive enzyme linked aptamers assay (ELIAA) for tetracycline was developed by Jheong et al. using both DNA and RNA aptamers (Jeong and Rhee Paeng, 2012). Zhou et al. fabricated a simple electrochemical tetracycline aptasensor with multi-walled carbon nanotubes modification. The electrochemical aptasensor exhibited a good sensitivity and was successfully applied to the determination of tetracyclines in real samples (Zhou et al., 2012).

Electrochemical aptasensors for toxins

Mycotoxins are the major group of toxins that are present in our food. They are group of naturally occurring chemicals produced by molds growing on different typed of crops. They have several adverse impacts on human health such as gastrointestinal diseases to kidney damage and immune suppression. Among the mycotoxins, Ochratoxin A (OTA) is the most commonly occurring toxins, and a DNA aptamer was selected against it in 2008 (Cruz-Aguado and Penner, 2008). Afterwards, numerous number of electrochemical aptasensors were developed to detect this toxins (Rhouati et al., 2013b). Bacterial endotoxins are also one of the major contaminants present in pharmaceutical products, causing sever septic shock in humans and animals (Magalhaes et al., 2007). Kim et al. fabricated an electrochemical aptasensors for the detection of endotoxins from crude biological liquor (Kim et al., 2012). Seeds of the leguminous herb lupin are the widely used source of low cost protein. However, there have been an increasing number of cases reporting severe allergic reactions to these seeds. To meet this challenge and detect Lupin allergen levels in food, a DNA aptamer based assay was developed by Nadal et al. (2012). Many toxins are excreted by human sand animals and subsequently entering into water effluents. Endocrine disrupting compounds form a major class of pollutants with several health hazards (Chang et al., 2009). Estradiol is one such

compound which has adverse effects on the male reproductive system. Recently, Yildirim et al. designed a DNA sensor based on fluorescence detection method for environmental water samples (Yildirim et al., 2012). However, this method can be adapted to the electrochemical methods for better analytical performance in future. Bisphenol A is used as a monomer constituent in plastic poly hydrocarbonate products. However, it has potential harmful effect for humans and animals as it interact with endocrine system by blocking of estrogen with its receptors. Jo et al. developed a sol gel biochip aptasensor to detect Bisphenol A in water samples. Further, electrochemical aptasensors can be designed to measure Bisphenol A based on the aptamer platform (Jo et al., 2011). Zhou et al. developed a simple and label-free electrochemical aptasensor for bisphenol A (BPA) determination. The method was based on the gold nanoparticles dotted graphene nanocomposite film modified glassy carbon electrode. The proposed aptasensor was rapid, convenient and low-cost for effective sensing of BPA (Zhou et al., 2014).

Electrochemical aptasensors against heavy metals

Heavy metals including mercury, arsenic.. etc. cause severe toxicity to nervous and endocrine system, and also heart problems, skin lesions, and cancer. According to official sources, arsenic level in water is exceeded than the allowable limit leading to serve arsenic toxicity among inhabitants. As a result, an aptasensor has been proposed by Kim et al. group to detect arsenic, with potential tool to be used as alarming method (Kim et al., 2009). Mercury has also been detected by the combination of gold nanoparticles and aptamer with optical output (Li et al., 2009; Helwa et al., 2012). However, the mercury specific aptamer can be very easily extended to electrochemical sensing platform in future. Li et al. developed an electrochemical DNAzyme sensor for sensitive and selective detection of lead ion (Pb^{2+}), taking advantage of catalytic reactions of a DNAzyme upon its binding to Pb^{2+} and the use of DNA-Au bio-bar codes to achieve signal enhancement. Although this DNAzyme sensor was demonstrated for the

detection of Pb²⁺, it has the potential to serve as a general platform for design sensors for other small molecules and heavy metal ions (Shen et al., 2008). Chen et al. reported a highly sensitive and specific electrochemical aptasensor for Cu²⁺ detection based on gold nanoparticles. Rapidity, simplicity, and excellent selectivity made it suitable for practical use in determination of Cu²⁺ from lake samples (Chen et al., 2011).

Till date, a number of aptamer based sensors have been developed for environmental applications to detect very low level of heavy metals. But still there are many toxic heavy metals for which aptamer have not been designed, so future work may focus to synthesize aptamer against the rest of toxin metal target analytes.

Electrochemical aptasensor for pesticides

Atrazine is one of the most intensively used pesticides to inhibit the growth of weeds. However, its presence may cause reproductive damage in humans. Sinha et al. screened a series of aptamers against atrazine and then cloned them into *E. coli* cells (Sinha et al., 2010). The screened aptamers may find potential applications in developing electrochemical aptasensors to detect atrazine in future. Similarly, insecticides are also applied to crops to protect them from insect attack. One such example is acetamipride, which when leached into the environment can cause toxicity in humans and animals. A series of aptamers which bind to acetamiprid with high dissociation constant have been synthesized recently (He et al., 2011; Wang et al., 2012). Wang et al. designed a DNA aptamer which was able to detect up to four highly poisonous organo-phosphorous pesticides including phorate, profenofos, isocarbophos, and omethoate (Shen et al., 2008). Fan et al. developed an aptasensor for sensitive and selective detection of acetamiprid based on electrochemical impedance spectroscopy (Fan et al., 2013). To improve sensitivity of the aptasensor, gold nanoparticles were electrodeposited on the bare gold electrode surface by cycle voltammetry. The applicability of the developed aptasensor was successfully evaluated by determining acetamiprid in the real samples, wastewater and tomatoes. Although aptamers have been selected against various types of pesticides, however, their potential as ligand molecules in a biosensor is mainly unexplored.

ELECTROCHEMICAL APTASENSORS AGAINST PATHOGENS

Detection, identification, and quantification of microbial pathogens are crucial for public health protection. Areas where detection of microbial pathogens is critical include water and environmental analysis. Aptasensors capable of rapidly detecting pathogens with improved analytical performance are highly desired to meet the increasing environmental problems. The aptamer based assays are able to identify and detect different types of pathogens without prior information of their membrane molecules and structural genes. Among the pathogens, aptamers assays against virus are in their infancy and some of the reported aptamers against specific viruses could get potential in the fabrication of biosensors. Various aptasensors platform have been reported in the literature for various types of viruses (Minunni et al., 2004; Tombelli et al., 2005; Lee et al., 2007; Tang et al., 2009). Similarly, detection of bacteria is also a relatively emerging and new area. Aptasensors based on different types of nanomaterials

in combination with electrochemical transduction output are described for the measurement of bacteria (So et al., 2008; Cella et al., 2010). Aptamer-functionalized single walled carbon nanotube field-effect transistor (SWNTFET) arrays aptasensor was developed to detect *E. coli* DH5 α (Fan et al., 2013). The binding between *E. coli* cells and the aptamer-functionalized FET resulted in a change in conductance of the samples which was subsequently related to detection mechanism. Recently, bioterrorism has been considered as a major threat to national security. One of the leading examples is Anthrax that is associated with the spores of gram positive bacteria *Bacillus anthracis*. Cella et al. reported an aptasensor for detection of protective antigen of anthrax (Tang et al., 2009). The aptasensor consisted of single stranded DNA aptamer functionalized to single walled carbon nanotubes having sensitivity in the nanomolar range.

CONCLUSION AND PERSPECTIVES

Despite of the attractive advantages, aptamer based assays are still under phase of development as compared to immunoassays for environmental monitoring. The primary hurdles are the limited number of available aptamers and relatively poor knowledge of aptamer immobilization strategies. However, recent years have witnessed important and rapid advances in the sequences of new aptamers, along with integration of new nanomaterials in aptamer based assays, rapidly improving the existing procedures. Electrochemical aptasensors reveal certain advantages when compared to the optical sensors. For example, the possibility of integrating signal amplifying catalytic or biocatalytic labels to the aptamer-target complex enables the highly sensitive detection of the target substrate. Recent advances have also witnessed the electrochemical label free detection, thus overcoming the requirement of labels. Even though substantial progress has been accomplished in the design of electrochemical aptasensors, several exciting, and improving opportunities still exist in the field of aptasensors. For example, the use of synthetically modified nucleotide as co-component and integration of the binding properties of aptamer with DNzyme may yield the hybrid structures with superior sensing functions by combining selected binding and catalytic properties of aptamer. Overall, the potential of electrochemical aptasensors is immense in the environmental monitoring, and this exciting and challenging area is on the brink of exponential growth. The future development in aptasensors with respect to environmental monitoring may focus on the design of aptamers against unexplored target analyte, and then their subsequent integration in the electrochemical platform to monitor those target analytes.

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Photosynthesis at the forefront of a sustainable life

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The development of a sustainable bio-based economy has drawn much attention in recent years, and research to find smart solutions to the many inherent challenges has intensified. In nature, perhaps the best example of an authentic sustainable system is oxygenic photosynthesis. The biochemistry of this intricate process is empowered by solar radiation influx and performed by hierarchically organized complexes composed by photoreceptors, inorganic catalysts, and enzymes which define specific niches for optimizing light-to-energy conversion. The success of this process relies on its capability to exploit the almost inexhaustible reservoirs of sunlight, water, and carbon dioxide to transform photonic energy into chemical energy such as stored in adenosine triphosphate. Oxygenic photosynthesis is responsible for most of the oxygen, fossil fuels, and biomass on our planet. So, even after a few billion years of evolution, this process unceasingly supports life on earth, and probably soon also in outer-space, and inspires the development of enabling technologies for a sustainable global economy and ecosystem. The following review covers some of the major milestones reached in photosynthesis research, each reflecting lasting routes of innovation in agriculture, environmental protection, and clean energy production.

Keywords: photosynthesis, photosynthetic yield improvement, bioremediation, biofuels, biosensors, artificial photosynthesis, sustainability, space agriculture

INTRODUCTION

Sustainability has been described by the European Union as follows: “Strictly speaking sustainability implies the use of resources at rates that do not exceed the capacity of the Earth to replace them.” (<http://ec.europa.eu/environment/eussd/food.htm>). Sustainability however does not come about by its own but requires dynamic and responsible actions to create and maintain a balance between society, environment, and economy. In the next 10–20 years, a steady increase of the global population, continuous competition for land, water, and energy, and the worsening effects brought by climate change will be the three foremost science policy-determining factors. Innovations in agriculture no doubt will have a great impact on sustainability since they would address the usage and purification of water, the availability of good-quality soil, the supply of energy including “green” energy, the importance of biodiversity, and above all, the command and safety of food and feed. It is mandatory however that this is done in an interdisciplinary way following a holistic approach.

Photosynthesis is one of the most efficiently cycled and sustainable processes we know in Nature. This deceptively simple process forms the basis for all the energy sources essential to life, from the intake of food to the burning of fossil fuels, and more recently, for the industrial production of value-added chemicals or bio-energy. Green plants, algae and cyanobacteria are able to

oxidise water for photosynthesis and hence are oxygenic, in contrast to other phototrophs that use different electron donors, such as hydrogen sulfide. The splitting of water (H₂O) giving off oxygen gas (O₂) is a complex event, requiring the absorption of solar energy by a set of aligned chlorophyll (Chl) pigments that as a result release electrons to convert CO₂ to carbohydrates, a reaction known as carbon fixation. The full process can be summarised in a single equation:



Oxygenic photosynthesis evolved approximately 2.5 billion years ago, the core reactions, light harvesting, charge separation, water splitting, and energy storage remaining similar across species. However, natural optimization of the mechanism through a series of fine physical and biochemical modifications allowed adaptation of the process to specific ecological niches. For instance, the evolution of C3 into C4 pathways enabled plants to attain, under certain conditions, an efficiency increase of their photosynthetic processes of up to 50%.

This review reports on research inspired by photosynthesis, addressing global, environmental and societal issues related to crops improvement, eco-system homeostasis maintenance and clean energy production, with the aim to identify opportunities and challenges for sustainable innovation and development.

PHOTOSYNTHESIS AT THE FOREFRONT OF A SECURE FOOD SUPPLY

The global demand of nutrition largely depends on photosynthesis efficiency. Presently achieved crop yields however lay far below the projected needs required to meet the predicted population growth, threatening global food security (Fedoroff et al., 2010; Long, 2014). Current photosynthesis research is much inspired by the call for a sustainable agriculture and the tuning of food, feed, and energy production in respect to each other (Nair, 2014). The main challenge however lays in increasing crop yields without encumbering land and water resources nor by burdening the environment with an excess of herbicides or nitrogen-rich manure. Analyses of current knowledge and outputs of biochemical and microclimatic photosynthetic models indicate that, by exploiting variations of existing germplasms and by engineering photosynthesis (Zhu et al., 2010a; Reynolds et al., 2011; Gu et al., 2014), sustainable yield increases of crops could be achieved. The present section encompasses recent advances to increase the radiation-use efficiency (RUE) of crops, hence production yields, by overcoming natural photosynthetic limits and improving light perception.

OVERCOMING THE MAIN LIMITS OF C3 PHOTOSYNTHESIS

Plant growth and biomass production rely on carbohydrate synthesis within the Calvin-Benson cycle, which is initiated by the incorporation of CO₂ into ribulose 1,5-bisphosphate (RuBP) by ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO). Eukaryotic RuBisCO comprises eight large (LS, chloroplast-encoded; containing the active sites) and eight small (SS, nuclear-encoded) subunits, organized in L₈S₈ macromolecules (Whitney et al., 2011a). RuBisCO catalysis requires pre-activation via an ancillary enzyme, RuBisCO activase (RA). It involves a complex 5-step reaction, and is further complicated by electrostatic similarity between CO₂ and O₂, as well as regular enzyme inhibition and -reactivation (Andersson and Backlund, 2008). RuBisCO promptly reacts with O₂, losing approximately 25% of previously fixed CO₂ through the photorespiratory pathway. RuBisCO's dual function and its low carboxylation rate (V_c) compromise its efficiency and are among the main factors determining the low RUE of C3 plants (Zhu et al., 2010b). Manipulation of RuBisCO's catalytic traits, modulation of its inhibition, or RA optimization were recognized as main targets in the struggle for the increase of crops yields (Raines, 2011; Whitney et al., 2011a; Parry et al., 2013; Mueller-Cajar et al., 2014). In addition, improving C3 RuBisCO catalytic turnover should diminish the required RuBisCO amount (normally representing approximately 50 and 25% of leaf protein and nitrogen content, respectively) increasing nitrogen use efficiency of crops.

Comparative analyses of natural RuBisCO variants demonstrated that an increase in the carboxylation rate is generally obtained at the expense of CO₂ affinity (K_m) and/or specificity ($S_{C/O}$). The high V_c of C4 type RuBisCO, coupled to low CO₂ specificity/affinity, requires restriction of the enzyme's access to atmospheric oxygen for best results. A more intriguing target is a variant RuBisCO which evolved in some red algae and possesses high $S_{C/O}$, keeping V_c values similar to those in the C3 plants (Whitney et al., 2011a). Another promising approach is a detailed

structural, biogenetic and catalytic characterization of RuBisCO forms developed under challenging growth conditions (Miller et al., 2013). The production of chimeric L₈S₈ complexes or manipulation of LS and SS is hindered by the complex RuBisCO biogenesis and spatial separation of the LS- and SS-encoding genes in the chloroplast and nucleus, respectively (Whitney et al., 2011a; Parry et al., 2013). However, a better understanding of RuBisCO folding and assembly (Liu et al., 2010; Kolesinski et al., 2013) has enabled the engineering of LS peptides with improved catalytic properties in *Chlamydomonas reinhardtii* (Zhu et al., 2010a; Maliga and Bock, 2011) and tobacco plants (Whitney et al., 2009, 2011b) resulting in increased photosynthetic rates and higher biomass. Furthermore, RuBisCO-mediated carbon fixation with C4-like catalysis (high V_c and high K_m) was realized in rice by introducing SS from sorghum and eliciting functional chimeric L₈S₈ complexes (Genkov et al., 2010; Ishikawa et al., 2011).

Due to the natural behavior of RuBisCO enzyme to form stable intermediate complexes with sugar phosphates, its V_c declines with time and repeated interventions of RA are required to prevent the so-called enzyme fallover (Mueller-Cajar et al., 2014). Attempts have been made to reduce RuBisCO fallover by lowering its sensitivity to the specific by-products or by accelerating the transformation of RuBisCO inhibitors into less active metabolites (Parry et al., 2013). Phosphate-induced impediment of RuBisCO heightens with the increase in temperature and highlights the main flaw of RA: its low thermostability. It was demonstrated that expanding the temperature range of RA stability (via overexpression of RA from warm- into cool-season species) or improving RA thermostability (via replacing the endogenous RA by a more thermostable enzyme) can give cause to increased photosynthetic performances and yields under moderate heat stress (Kumar et al., 2009; Carmo-Silva and Salvucci, 2012).

Metabolic control analyses indicated that the RuBisCO carboxylation reaction is the controlling step in the efficacy of the Calvin-Benson cycle, particularly under high light, high temperature and low CO₂ conditions (Zhu et al., 2010b; Raines, 2011). Investigations using antisense RNA silencing to reduce RuBisCO protein levels revealed additional enzymes able to control the C3-cycle efficiency by modulating the RuBP regeneration rate (Raines, 2011). However, the mechanisms that regulate carboxylation-cycle reactions must be fully understood in order to introduce appropriate modifications. It was shown that the increase in photosynthetic rate and concomitant gain in biomass owing to the overexpression of sedoheptulose-1,7-bisphosphatase in fact is highly species- and growth dependent and that the overexpression of transketolase actually affects plant growth negatively, probably due to changes in the C3-cycle carbon exchange balance (Raines, 2011).

Parallel to the efforts to engineer RuBisCO proteins with increased V_c , extensive research focused on increasing C3-cycle productivity by bypassing the photorespiratory pathway. Photorespiration entails the sequential transformation of phosphoglycolate (PG), the RuBP oxygenation product, back into glycerate to join the Calvin-Benson cycle in the chloroplast via a series of peroxisome- and mitochondrion-located reactions (Peterhansel et al., 2010). The regeneration of oxygenated RuBP

consumes reducing equivalents and energy, and is associated with the release of CO₂ and ammonia derived from previously fixed carbon and nitrogen (Peterhansel et al., 2013). Three different approaches to circumvent this “wasteful” lane have been proposed and tested in plants with different successes (**Figure 1**): (i) catabolization of PG to RuBP and CO₂ in chloroplasts (Kebeish et al., 2007) (Bypass 1), (ii) a similar reaction in peroxisomes (Carvalho et al., 2011) (Bypass 2), and (iii) oxidization of PG to CO₂ and pyruvate in chloroplasts (Maier et al., 2012) (Bypass 3). Bypasses 1 and 3 proved to be functional in *Arabidopsis* plants, and resulted in enhanced photosynthesis and growth. Both pathways are energetically less costly than the photorespiratory route, possibly avoiding ammonia re-fixation and releasing CO₂ in chloroplasts (Peterhansel et al., 2010; Maier et al., 2012; Peterhansel et al., 2013). The integration of these basic approaches into agriculturally significant crops is still a challenge and the translation of their benefits into crop yields needs to be determined.

Some green algae and cyanobacteria are useful as feedstock and are regarded as safe for human consumption. Such microalgae generally perform C3 photosynthesis and rely on bicarbonate and CO₂ transport. A carbon concentration mechanism (CCM) in green algae consists of a spatial regulation of carbonic anhydrase activity and the low-CO₂ induced formation of a starch sheet around the pyrenoid, a proteinaceous structure where most

(>90%) of the RuBisCo becomes encapsulated. In cyanobacteria a similar CCM exists, but here bicarbonates are transferred from the cytoplasm to a separate compartment, the carboxysome, which is not permeable by oxygen. Herein, a carbonic anhydrase converts bicarbonate to CO₂ used as enriched substrate by the carboxysome-enclosed RuBisCo. It has been recently suggested that C3 plant photosynthesis might be improved through the introduction of microalgal bicarbonate pumps (Price et al., 2013) or complete carboxysomes (Zarzycki et al., 2013) or pyrenoids (Meyer and Griffiths, 2013) into their chloroplasts but these potential routes have not yet been demonstrated.

ENGINEERING C4 PHOTOSYNTHESIS IN C3 CROPS

Today, although only 3% of all vascular plants exhibit C4 photosynthesis, they account for one-fifth of the global primary productivity owing to ample high-yield C4-grass-lands. In general, C4 plants (i.e., maize, sugar cane, sorghum) are more efficient at photosynthesis than C3 plants and fix carbon at higher rates, use less water per weight of biomass produced, and tolerate to a better extent water and high temperature stress. But, in terms of ground use and taking into account that only parts of plants are edible, C3 crops (e.g., potato, soybean) produce some of the highest edible calories and protein per square meter. C4 photosynthesis is characterized by a two-step carbon fixation for the establishment of a CO₂-enriched RuBisCO environment and

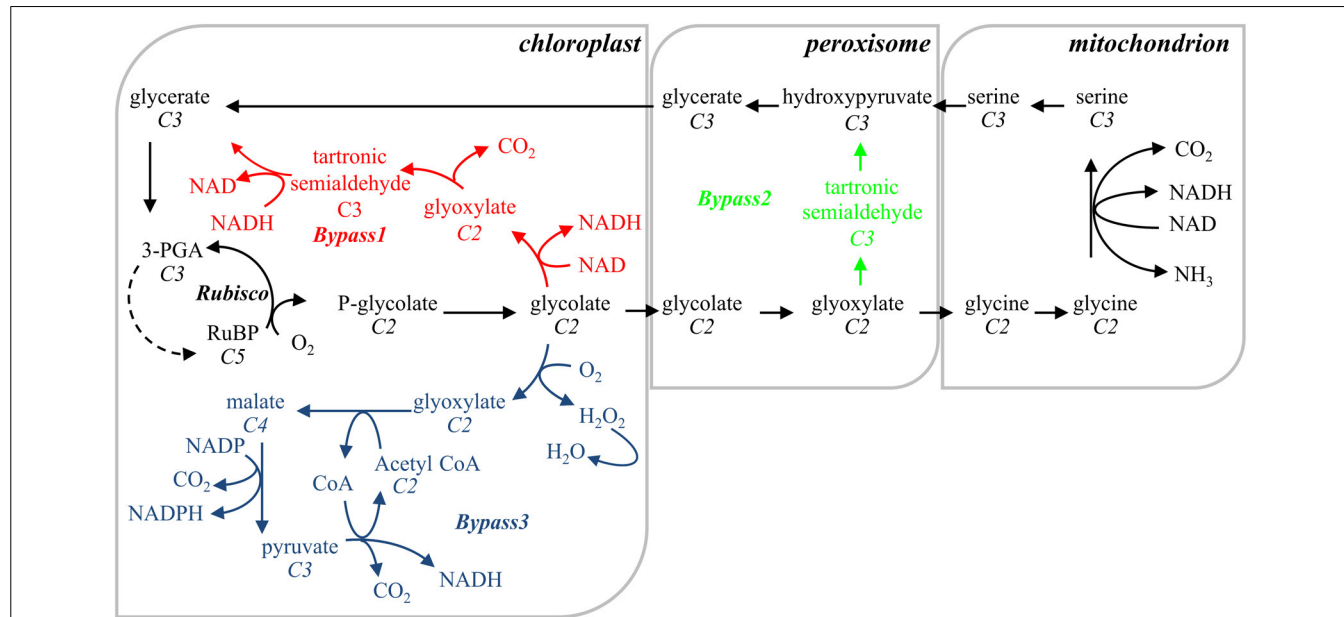


FIGURE 1 | Schematic representation of the photorespiratory pathway (in black) and the three circumvent pathways designed to overcome the photorespiratory losses. The reactions of bypass 1 (in red) are entirely realized into the chloroplast and comprise the transformation of glycolate to glycerate, introducing glycolate dehydrogenase, glycine decarboxylase and tartronate semialdehyde reductase similar to the *E. coli* glycolate catabolic pathway (Kebeish et al., 2007). Bypass 2 (in green) follows the *E. coli* glyoxylate catabolic pathway in the peroxisomes by introducing glycine decarboxylase and hydroxypyruvate isomerase (Carvalho et al., 2011). Bypass 3 (in blue) oxidizes glycolate to CO₂ in the chloroplast,

using exogenous (glycolate oxidase and catalase from the peroxisomes, and malate synthase from the glyoxysomes) and endogenous (malic enzyme and pyruvate dehydrogenase) enzymes (Maier et al., 2012). In all three bypasses release of ammonia in the mitochondrion is abolished; 75% of the glycolate redirected toward bypasses 1 and 2 is returned to the Calvin-Benson cycle as 3-PGA; bypasses 1 and 3 dislocate CO₂ released from the mitochondrion to the chloroplast. Reactions stoichiometry is not taken into account; the numbers of carbon atoms of each metabolic compound are in italic; 3-PGA, 3-phosphoglycerate; RuBP, ribulose 1,5-bisphosphate.

creates conditions in which photorespiration is negligible. The majority of C₄ species have higher RUE and biomass production than the C₃ types, particularly in warm habitats (Langdale, 2011). These benefits are especially evident in species which evolved CCM, spatially separating the carbon fixation and carboxylation between the chloroplasts of mesophyll cells (MCs) and bundle sheath cells (BSCs), also known as Kranz anatomy or the Kranz-type C₄ cycle. In contrast to C₃, both C₄ cell types are rich in chloroplast: MC chloroplasts possess a granal-stromal lamellae organization and lack the enzymes involved in the Calvin-Benson and photorespiration pathways; BSCs show predominantly stromal lamellae and a typical C₃ enzymatic profile. C₄ metabolism invests in enlarged and photosynthetically active BSCs, a high number of veins, a heavy metabolite traffic, and the functional coordination between BSCs and MCs. The introduction of a C₄ cycle into typical C₃ species, such as rice and soybean, is predicted to increase crop yield, with concomitant improvement in water and nitrogen-use efficiency (Zhu et al., 2010b; Langdale, 2011; Wang et al., 2011). The difficult transfer of high-yield C₄ metabolism in important C₃ crops, such as rice, became one of the main goals of the international C₄ Rice Consortium (<http://c4rice.irri.org/>) established less than 10 years ago and aiming to address the growing food demand (von Caemmerer et al., 2012). The production of rice with acquired functional C₄-biochemistry requires modification and integration of numerous biochemical pathways and adequate adaptation of rice leaf anatomy. The main approaches undertaken for the realization of this ambitious project have been thoroughly described (Kajala et al., 2011; Langdale, 2011; Leegood, 2013; Karki et al., 2013) and include: (i) the integration in rice of genes typical for the C₄ metabolism, reaching expression levels conferring high photosynthetic yields, such as the genes encoding phosphoenolpyruvate carboxylase in MCs and enzymes from Calvin-Benson cycle in BSCs, (ii) the down-regulation of endogenous rice genes, e.g., encoding MC enzymes of the Calvin-Benson cycle and photorespiration, (iii) the introduction of C₄ cell-type specific gene expression and protein accumulation in rice, including identification of suitable regulatory elements to ensure the protein's compartmentalization between MCs and BSCs, and (iv) the identification of C₄ transporters carrying out the metabolite transfer between the subcellular compartments and the introduction of the corresponding genes into the rice genome. Significant progress was made through the identification of gene promoters for compartmental gene expression (Wang et al., 2013a), gene cloning for the main enzymes of the C₄ metabolic pathway from maize and their transformation into rice (Kajala et al., 2011), and the determination of candidate transporters of intermediate metabolites between MCs and BSCs (Karki et al., 2013).

Another important challenge is altering rice leaf anatomy and morphology in order to make it comparable with the Kranz-type biochemistry. The BSCs of rice, a typical C₃ plant, contain too few chloroplasts to attain high-yield C₄ photosynthesis (Karki et al., 2013). This hurdle can be bypassed by cell-type specific overexpression of genes implicated in chloroplast development, thylakoid stacking, and photosystems assembly such as the homologous gene pair of the *Golden2-like* (*GLK1*, *GLK2*) transcription factors (Langdale, 2011; Karki et al., 2013). The

confirmation of a differential accumulation of GLKs in maize MCs and BSCs and their regulatory role in dimorphic chloroplast differentiation (Wang et al., 2013b) proved this approach to be correct. Even more encouraging was the successful introduction of Kranz anatomy traits in C₃ leaves, which caused an increase in vein density and larger BSC cells in an oat-maize chromosome addition (OMA) line (i.e., *Avena sativa* plants containing a maize chromosome) (Tolley et al., 2012). A large interaction surface and closed contact between MC and BSC are mandatory requirements for fulfilling the needs of the heavily loaded intercellular metabolite exchange typical for the C₄ cycle. Recently, the first gene (*scarecrow*) responsible for the C₄ specific leaf patterning could be identified (Slewiniski et al., 2012; Wang et al., 2013a) while a better knowledge of Kranz-type leaf structure development and the underlying evolutionary mechanisms will be instrumental to engineer high-yield photosynthesis in rice (Slewiniski, 2013).

IMPROVING CANOPY PHOTOSYNTHETIC PERFORMANCE

Improvements of the photosynthetic efficiency on chloroplast/leaf level can lead to an increase of crop yield if the engineered traits can be effectively introduced on plant canopy level. In this context, the main concern is the unequal light distribution within the plant canopy, resulting in an excessive light overload at canopy surface and severe light limitation within the lower canopy levels (Zhu et al., 2010b, 2012). Improving the efficiency in which crops are able to convert intercepted light into biomass requires the identification of an ideal canopy architecture (thus optimizing canopy performance) while keeping in mind the species- and habitat-specific requirements. A possible approach is the breeding or engineering of plants with more erected leaves and dwarf phenotypes causing a better canopy light distribution and conversion while reducing stem investment and lodging losses. Mathematical models can estimate quite accurately the canopy distribution of the light-limited and light-saturated photosynthesis in different species and environmental conditions, thus assisting the identification of improved crop specific architecture (Zhu et al., 2012; Song et al., 2013). To this end, considerable progress was made in unraveling the genetic basis of plant architecture (Jin et al., 2008; Wang and Li, 2008; Zheng and Liu, 2013).

Additional tactics to improve canopy RUE have also been explored. One of the most commonly used approaches is the reduction of leaf chlorophyll content, i.e., by light-harvesting antenna complex (LHC) size reduction, to effectively diminish excess light caught by upper-canopy leaves. The superfluous absorbed light causes a photoinhibitory inactivation of the photosynthetic reactions and is largely dissipated through non-photochemical quenching mechanism. In addition, this approach would cause an increase in light availability in the lower-canopy levels, relieving their light-limited photosynthesis (Zhu et al., 2010b; Ort et al., 2011). Furthermore, increased canopy light penetration could also positively alter the heat canopy balance by lowering the temperature in the upper portion and increasing it near to the soil, thus improving crop yield (Ort et al., 2011).

The adjustment of chlorophyll content and composition as well as truncation of light-capturing antenna has been successfully applied for an improvement of RUE and an increase in

biomass yield for microalgal mass cultures. Among the different strategies to accomplish this, silencing or down-regulation of LHC encoding genes proved to be effective, leading to a higher cell culture density, less fluorescence quenching and a better photosynthetic quantum yield, while algal cultures were more tolerant to oversaturating light intensities (Kirst et al., 2012; Gimpel et al., 2013). In contrast, *Synechocystis* sp. PCC 6803 mutants with truncated phycobilisome antenna showed reduced growth rate and whole-culture biomass production (Page et al., 2012; Liberton et al., 2013). For crops, the benefits of LCH size reduction in terms of season-long carbon gains have yet to be rigorously tested (Ort et al., 2011). Other lines of interest are the discovery of the hitherto unknown chlorophyll *f* displaying a red-shifted absorption maximum (Chen et al., 2010) and the reconsideration of the photosynthetic potential of chlorophyll *d* (Mielke et al., 2013). These findings propounded the idea to improve plant RUE by extending the by plants usable spectrum of photosynthetically active radiation, or PAR (Chen and Blankenship, 2011; Blankenship and Chen, 2013). Besides the development of all these strategies to boost high photosynthetic yields, the possibility to improve photoprotection capacity in crops should be kept in mind as well (Murchie and Niyogi, 2011).

BIOREMEDIATION AND BIOMONITORING BASED ON PHOTOSYNTHESIZERS

Synthetic fertilizers and agrochemicals play a major role to meet the ever increasing global demand for food. However, the excessive and improper use of these chemicals has turned agriculture into a major source for environmental pollution. A fine example is the use of nitrogen-rich fertilizers which, while allowing for a tremendous increase in crop productivity, resulted in nitrate and nitrite run-offs that have heavily disturbed many lake and river ecosystems. In addition, the ammonium required for fertilizer production is industrially made via the Haber process utilizing up to 5% of the global natural gas production. Thus, the current use of such fertilizers translates in massive CO₂ emissions arising from the nitrogen extraction, the transport of raw materials and products, as well as the actual fertilizer production.

Adjusting fertilizer input to avoid excessive runoffs and preserve fossil gas resources have become priorities for modern farming practices. In particular, the development of field sensing methods, such as deployed for nitrate (Plumeré et al., 2012a,b; Plumeré, 2013), may allow farmers to better estimate the fertilizer needs of crops. In parallel, remediation of contaminated water, soil, and atmosphere has become exigent. Commonly used nitrate removal methods include chemical precipitation, ion exchange, electrodialysis, and reverse osmosis. While these strategies may be applied to clean up contaminated water, they are impractical for *in situ* use, come at an additional energy cost, and cause further CO₂ emissions. Instead, solar light driven microbial processes are applicable *in situ* and carry a greater promise for environment-friendly bioremediation.

The general term bioremediation refers to a number of waste management techniques involving the use of plants or microbes (bacteria, yeasts, fungi, algae) to eliminate or reduce the concentration of pollutants from a contaminated site (reviewed by Bhatnagar and Kumari, 2013; Willscher et al., 2013). Lately,

the term phytoremediation has been adopted for the specific use of plants, while the term phycoremediation is now specifically applied to the use of (green) algae or cyanobacteria. Phytoremediation may consist of one or more of six different phytotechnologies (phytotransformation, rhizofiltration, phytostabilization, phytovolatilization, evapotranspiration, and phytoextraction) depending on the used plant and the type and depth of contamination (Paz-Alberto and Sigua, 2013; Moosavi and Seghatoleslami, 2013). Phycoremediation equally benefits from the use of oxygenic photosynthesis but the use of microalgae and cyanobacteria offers some advantages including a higher biomass productivity, a much faster growth, an easier control of cellular response, the avoidance of arable land use, and the ability to extract micro- and macronutrients from wastewaters or industrial flue gasses (Anemaet et al., 2010; McGinn et al., 2011; Pittman, 2011). Plants, microalgae, and cyanobacteria have been studied extensively in recent years as effective accumulators, biosorbents, and degraders useful for the bioremediation of different kinds of organic and inorganic pollutants (Olguín, 2003; Gosh and Singh, 2005; Mehta and Gaur, 2005; Marmiroli et al., 2006; Lone et al., 2008; Chinnasamy et al., 2010; Kong et al., 2010; Wang et al., 2010; De Philippis et al., 2011; Moosavi and Seghatoleslami, 2013; Paz-Alberto and Sigua, 2013).

Coupling the growth of microalgae on wastewater with energy production has been proposed since the 60's (Oswald and Golueke, 1960; Benemann et al., 1977; Hoffmann, 1998; Mallick, 2002; Rawat et al., 2011; Olguín, 2012; Fahti et al., 2013) while the efficiency of sustainable low-cost wastewater treatment based on microalgae has been confirmed in the past decade (de-Bashan and Bashan, 2010). In spite of these promising developments, several challenges and limitations exist for microalgal biomass in terms of aeration and adequate quantities of light (taking into account self-shading and turbidity in ponds or reservoirs, but also photoinhibition), fluctuations in temperature and pH, harvesting and extraction costs, and down-stream processing (Scott et al., 2010; Hannon et al., 2011; McGinn et al., 2011; Rawat et al., 2012). Yield and cost analyses for algal systems versus traditional fuel crops indicate that algal systems are not yet cost-effective (van Bellen, 2009; Beal et al., 2012; Slade and Bauen, 2013) and life cycle assessments (LCAs) such as recently reported by Passell et al. (2013) using commercial data for algal production of biodiesel are clearly wanted (see also section Evaluation of Environmental and Social Impacts of Biomass Energy production). Nonetheless, microalgal, and cyanobacterial systems that combine bioremediation (i.e., waste water treatment) or CO₂ mitigation with the production of potentially valuable biomass—whether biofuels or other added-value products—remain attractive novel routes. In particular when (bio)technological advances, including genetic modifications, could improve their cost-effectiveness and enhance their role in global endeavors for a sustainable life. Hence, in the next sections of this review we mainly focus on microalgal bioremediation and biofuels.

MICROALGAE FOR BIOREMEDIATION

Phosphorus and Nitrogen removal

Nitrogen and phosphorus are serious pollutants accumulating in waters as a result of agricultural runoff. The major effect

of releasing wastewater rich in organic and inorganic chemicals such as nitrates and phosphates is mainly eutrophication (Correll, 1998), with consequent hypoxia or anoxia of aquatic animals. Microalgae, in this context, can offer an attractive solution as they are able to grow in wastewater conditions by utilizing the abundant organic carbon and inorganic nitrogen and phosphorus (Pittman, 2011) thus acting as bioremediators against these elements.

Phosphorus. Phosphorus is an essential element for all life forms. Autotrophs can assimilate this mineral nutrient only as an orthophosphate, i.e., after hydrolysis of its organic forms by extracellular enzymes. The presence of this element in soils is often limited owing to the formation of insoluble complexes. In general, water-soluble phosphate used in fertilizer suffuses, while less than 20% is absorbed by plants (Vance et al., 2003). Phosphate enters ground water, streams and rivers, and moves out to sea and oceans where it is directly consumed by marine phytoplankton thus entering the food chain (Baturin, 2003). Since microalgae accumulate phosphorus as polyphosphate bodies stored inside the acidocalcisome organelle (Seufferheld and Curzi, 2010), these photosynthetic organisms can be doubly useful: as bioremediators, to remove the excess of phosphorus from waters, and as temporary storage, to capture this macronutrient and return it to the terrestrial environment in form of agricultural fertilizer (Sivakumar et al., 2012).

Nitrogen. Plants, microbes and algae absorb nitrogen from soil or water and store it as biomass. Over time, the biomass decomposes releasing nitrogen into the soil (e.g., as ammonia, urea) or into the atmosphere (e.g., as N_2O), where it may be recycled or lost. Although N_2O is not produced in significant amounts in the presence of nitrates, it is not known if other nitrogenous compounds found in wastewater, such as urea and ammonia, are converted to this greenhouse gas. Since microalgae have unique natural mechanisms for removing excess of nitrogen, phosphorus and CO_2 from water sources, these organisms have been widely investigated for nitrogen removal. *C. vulgaris* was used for nitrogen and phosphorus removal from wastewater with an average removal efficiency of 72% for nitrogen and 28% for phosphorus (Aslan and Kapdan, 2006), while other microalgae widely used for nutrient removal were *Chlorella* (Lee and Lee, 2001), *Scenedesmus* (Martínez et al., 2000), and *Spirulina species* (Olguín et al., 2003), next to *Nannochloris* (Jimenez-Perez et al., 2004) and *Botryococcus braunii* (An et al., 2003).

CO₂ capturing

Microalgae have the ability to fix CO_2 (generally via the Benson–Calvin cycle) with an efficiency 10–50 times higher than that of terrestrial plants (Li et al., 2008). Through the photosynthetic process, microalgae can completely recycle CO_2 , producing the chemical energy necessary for the completion of their vital functions. For this reason, CO_2 mitigation by microalgae is still considered the best strategy for an efficient removal of this greenhouse gas and to address global warming, especially when combined with algal biofuel production (Wang et al., 2008).

Light intensity, temperature, and CO_2 concentration strongly affect CO_2 fixation, in the way that increasing light intensity

while maintaining moderate temperature and moderate solute concentration, increases both fixation and CO_2 solubility in liquids (Atkinson and Mavituna, 1991). One of the best sources of highly enriched CO_2 is flue gas containing 10–20% CO_2 from burning fossil fuels (Ge et al., 2011), but algal species differ in their apparent ability to use CO_2 effectively. High level of CO_2 inhibit some species, while others can thrive on CO_2 levels up to 20% (certain strains of *Chlorella*, *Scenedesmus*, and *Cyanidium* even grow in 40–100% CO_2 —reviewed by Salih, 2011). The efficient mass transfer of CO_2 to cells in the aqueous environment of large-scale liquid culture systems is therefore challenging. Nonetheless, efficient capture of CO_2 , NO , and SO_2 for algal biomass production by directly introducing flue gas into microalgal cultures have been reported (Chiu et al., 2011 and reviewed by Van Den Henden et al., 2012).

Heavy metals

Heavy metals are known to cause, in human beings, various physiological disorders to hepatic, renal, respiratory, and gastrointestinal systems. The toxicity of heavy metals depends on their concentration, bioavailability, and chemical forms, and the duration of exposure. The ever-increasing contamination of aquatic bodies and soils by heavy metals is an issue of serious concern and challenge world-wide. Bioremediation of heavy metal-contaminated water employing various microorganisms, including microalgae, has been recognized as a cheaper, more effective and an eco-friendly alternative to the conventional physico-chemical remediation methods. Considerable research effort has been therefore devoted to the development of algal biosorbents able to remediate these pollutants (Hazrat et al., 2013).

Biosorption of metal ions from aquatic complex matrices is based on the interaction of metal ions with the functional groups on the surface or within the cellular wall of the algae biomass. This phenomenon clearly depends on the typical binding profile of the biosorbent. Therefore, different algal species having different sizes, shapes, and cell wall compositions will have different metal binding efficiencies (Monteiro et al., 2011).

Current methods used to treat heavy metal wastewater include chemical precipitation, ion-exchange, adsorption, membrane filtration, coagulation-flocculation, flotation and electrochemical methods, even if only the first three techniques are the most frequently studied, as recently reviewed by Fu and Wang (2011) and Vandamme et al. (2013).

Cyanobacteria that produce extracellular polysaccharides (EPS) have been successfully applied for the removal of a wide range of metals from water, including Co, Cu, Cr, Pb, and Zn (reviewed by De Philippis et al., 2011) and extracted biomasses of cyanobacteria have been used to adsorb radionuclides of Cs, Sr, Ra, and Am (Pohl and Schimmack, 2006). An interesting application of green microalgae (*Hydrodictyon sp.*, *Oedogonium sp.*, and *Rhizoclonium sp.*) for the bioremediation of heavy metals such as As and Cd, is described by Saunders et al. (2012), who reported an alternative and practical approach to algal bioremediation of metals in which algae are cultured directly in the waste water stream. Other green algae, i.e. *Closterium moniliferum* and *Coccomyxa actinabiotis*, which show efficient and selective radionuclide sequestration and are extremely radioresistant, are

prime candidates for in situ biodecontamination in the nuclear industry (Krejci et al., 2011; Rivasseau et al., 2013). In the aftermath of the Fukushima nuclear accident the search for additional cyanobacteria and algae, but also aquatic plants, that can be deployed to efficiently eliminate radionuclides from the environment, has intensified (Fukuda et al., 2014). Another interesting study describes a system dynamics approach to explore the efficacy of using mixed microalgae populations to treat leachate-hypersaline water (Richards and Mullins, 2013). This model evaluates the temporal evolution of metal removal and lipid production using four common marine microalgae species (*Nanochloropsis*, *Pavlova lutheri*, *Tetraselmis chuii* and *Chaetoceros muelleri*) and shows that after a ten-day period, the microalgae population is able to remove over 95% of the metals from the solution, paving the way for new strategies of waste stream management based on the use of microalgae-based bioremediation coupled with lipid-production.

Remediation of solid-waste and wastewaters

Microalgae are also efficient agents for the assimilation of organic matter from various contaminated media. Different photosynthetic organisms, often microalgae/bacteria consortia, have been successfully used in the remediation of solid-waste and wastewaters containing pesticides (González et al., 2012; Jin et al., 2012), phenols (Chiaiese et al., 2011; Maza-Márquez et al., 2014), aromatic hydrocarbons (Ibraheem, 2010; Ghasemi et al., 2011), textile dyes and detergents (Sing-Lai Lim et al., 2010; Singh and Patel, 2012), primarily due to their capacity to metabolize these compounds as nitrogen, phosphorus, carbon, and sulfur sources. For detailed description of the mechanisms involved in the bioremediation of each aforementioned class of pollutants see McGinn et al. (2011).

Photosynthesis-based biosensors

Real-time monitoring of crop growth parameters and environmental field conditions are mandatory for the development of tailored-made strategies aimed at minimizing resource inputs while maximizing output and yield. In this context, biosensor technology revealed a more suitable tool compared to analytical conventional methods requiring sample pre-treatments and complex instrumentation.

Photosynthetic microorganisms offer versatile solutions for the construction of smart and sensitive biosensors, enabling the detection of even minute amounts of pollutants. The functional principle of these sensors relies on the interaction of some classes of herbicides, pesticides, or heavy metals with a specific pocket of the photosynthetic reaction centers. This evokes physico-chemical changes that can be easily converted into measurable electrical signals. For instance, mercury with a 10^{-14} – 10^{-6} M concentration range in industrial and agricultural effluents could be detected using *Chlorella* whole cell based biosensors (Singh and Mittal, 2012). Researchers have also shown that His-tag-purified reaction centers of *Rhodobacter sphaeroides* attached to a gold electrode are particularly suitable for specific biosensing of herbicides, as photocurrent generation was inhibited in a concentration-dependent manner by the triazines atrazine and terbutryn with a Limit of Detection (LOD) of 50 and 8 nM,

respectively, but not by nitrile or phenylurea herbicides, opening up suitable protein engineering approaches to develop more sensitive and more selective biosensing devices for the control of weeds (Swainsbury et al., 2014).

Wild-type and genetically engineered strains of the unicellular green alga *Chlamydomonas reinhardtii* were exploited to develop a set of portable and easy-to-use biosensors. These sensors, making use of photosynthetic biorecognition elements, enabled fast and low-cost pre-screening of triazines, diazines, and ureas in water samples (Buonasera et al., 2009; Scognamiglio et al., 2009). Protein engineering and synthesis of biomimetic peptides also allowed the design of *C. reinhardtii* mutants or novel photosynthetic binding domains with enhanced stability or tolerance to free-radicals-associated stress and heightened sensitivity for triazinic and ureic herbicides (with an LOD in the range of 10^{-8} M) (Rea et al., 2009; Lambrevia et al., 2013; Scognamiglio et al., 2013).

THE USE OF "NATURAL PHOTOSYNTHESIS" IN SOLAR-ENERGY-CONVERTING TECHNOLOGY BIOMASS ENERGY: A CARBON NEUTRAL RESOURCE

The extensive global use of fossil fuels greatly increased the release of CO₂ and other greenhouse gases into the atmosphere leading to important climate changes. There is a dire need for alternative energy sources (Frölicher and Joos, 2010; van Kooten, 2013) and hence biofuels produced from photosynthetic organisms or organic wastes offer the great opportunity to address the world's dependence on oil and to reduce CO₂ emissions.

While the burning of fossil fuels increases the CO₂ levels in the atmosphere by releasing carbon sequestered millions of years ago, the use of biomass maintains a closed carbon cycle returning carbon previously incorporated by growing plants to the atmosphere (Abbasi and Abbasi, 2010; Kopetz, 2013). Different biomass sources show large variations in terms of yield, quality, and cost. In the past, biomass from food crops, hydrocarbon-rich plants, waste reuse, or weed and wild plants were investigated for energy production and it was shown that the efficiency of mass-to-energy conversion is related to their biomass composition, i.e., the quantitative proportion between the three main organic constituents cellulose, hemicellulose, and lignin (Irmak et al., 2013; Zeng et al., 2014).

Recently a new energy production line from biomass derived from photosynthetic microorganisms (e.g., algae) has been added to the already known carbon neutral methodologies. While there are still challenges, the results obtained so far show the potential of this innovative approach (Ghasemi et al., 2012; Menetrez, 2012; Adenle et al., 2013).

BIOFUELS GENERATIONS

The history of biofuels starts with Louis Pasteur in the 19th century who in 1861 observed butanol production from anaerobic fermentation (later on picked up by Chaim Weizmann in 1913 studying acetone-butanol-ethanol fermentations in clostridia growing on a large range of biomass, most notably molasses). In the 1890s, Dr. Rudolf Diesel invented his revolutionary engine designed to run on a wide range of fuels, including vegetable oils, followed by Nicolaus Otto's pioneering spark-ignition engine

designed to run on ethanol (i.e., derived from plant mass). In the early 20th century petroleum became widely available and the biofuel concept gained little attention except for brief interests during World War II and the 1970s oil crisis. During the last few decades, interest for alternative energy sources was rekindled and different biofuels were introduced on the markets (Kovarík, 2013).

Current biofuels are classified depending on the feedstock type used. The first generation of biofuels, of which ethanol and biodiesel are the main exponents, are essentially derived from food crops such as soybean, wheat, sugar-cane, and corn, using different procedures depending on the type of “green fuel” to be produced (Lee and Lavoie, 2013). The production of ethanol is generally obtained by fermentation of C_6 sugars (e.g., glucose) through the action of yeasts, such as *Saccharomyces cerevisiae*, while biodiesel production requires a chemical process (Park et al., 2013). The treatment includes the extraction of the oil fraction from the edible biomass and its transformation in biodiesel through trans-esterification in the presence of methanol to obtain methyl esters (biodiesel), with glycerol as a by-product. Eventually the green fuel is recovered by repeated washings with water to remove glycerol and methanol (Naik et al., 2010; Lee and Lavoie, 2013).

Despite environmental benefits, the first generation of biofuels was accompanied by concerns about potential drawbacks generated by the competition with food and fiber products as well as by the competition for land and water (Ajanovic, 2011; Lee and Lavoie, 2013; Mohr and Raman, 2013), rising doubts about costs and sustainability and prompting research into the second generation of biofuels.

The second generation of biofuels exploits the potential of cheap, plentiful, non-edible biomass of lignocellulosic nature. This biomass is divided in three subcategories: homogeneous (e.g., wood chips), quasi-homogeneous (e.g., agricultural or forest residues) and non-homogeneous (e.g., municipal wastes) (Naik et al., 2010; Lee and Lavoie, 2013). The production is based on thermochemical or biochemical processes generating different end-products.

The thermochemical pathway transforms the whole biomass into three phase fractions by heat treatment in the presence of different oxygen concentrations: solid (biochar), liquid (bio-oil) and gaseous (syngas), their relative percentage being dependent on the different thermal conditions applied. At low temperatures (250–350°C) and in the absence of oxygen, torrefaction occurs and mainly biochar is obtained; at higher temperatures (550–750°C) and in the absence of oxygen, bio-oil is primarily produced through pyrolysis; at very high temperatures (750–1200°C) and in the presence of traces of oxygen, a gasification process occurs, producing mostly syngas. In theory any lignocellulosic biomass can be treated with any of the aforementioned thermo-chemical processes. In practice however technical and economic restrictions need to be considered on a case to case basis (Abbasi and Abbasi, 2010; Naik et al., 2010; Gallagher and Murphy, 2013; Lee and Lavoie, 2013).

The biochemical approach, exclusively applicable on the cellulosic fraction of the biomass, consists of hydrolysis, fermentation, and product separation. The complexity of this method

is due to the resistant nature of cellulose (usually requiring a pre-treatment), the plethora of sugars released after breakage, and the need of specific microorganisms (including genetically engineered ones) to obtain an efficient fermentation (Abbasi and Abbasi, 2010; Lee and Lavoie, 2013).

When it became apparent that biofuels based on (micro)algal biomass could potentially provide much higher yields with lower resource inputs, efforts concentrated on the third biofuel generation. Current microalgae culture systems exist as expensive photo-bioreactors or in weather-dependent and contamination-prone outdoor systems (Chen et al., 2011; Halim et al., 2012; Makareviciene et al., 2013), but they do not need valuable farmland and their impact on fresh water resources are minimal if water is recycled or when waste waters or industrial effluents are used. Their potential is also given by an excellent photosynthetic performance, a good tolerance to hostile environmental conditions (Singh et al., 2011a,b; Larkum et al., 2012; Allen et al., 2013; Borowitzka and Moheimani, 2013), and a great variety in content and lipid profiles depending on the used species, growth conditions, and medium composition (Halim et al., 2012; Nascimento et al., 2013).

The conversion process from (micro)algal biomass to biofuel starts with harvesting and dehydration. Collection can be realized by centrifugation, filtration, or flocculation. The latter method, having the lowest energy cost, is usually achieved through the addition of polymers to the suspension (Vandamme et al., 2013). After removal of most of the liquid, a pre-treatment (cellular decomposition by high-pressure homogenization, total dehydration, and milling to fine powder) is required to optimize the material for lipids extraction (Halim et al., 2012; Pahl et al., 2013) carried out by using organic solvents or supercritical fluids (e.g., highly pressurized liquid carbon dioxide—see below). Eventually the mixture is filtered to remove cellular debris, while extraction solvents and residual water are eliminated by distillation, vacuum evaporation or solid-phase solvent adsorption. The final product consists of a lipid crude extract, usually containing polar and non-acylglycerol lipids. Before trans-esterification, the fraction of non-acylglycerol lipids, considered a contaminant in the biofuels production, is removed by liquid chromatography, acid precipitation and urea crystallization. During the trans-esterification, fatty acids are converted to alkyl esters in the presence of alcohols. The final mixture is purified to remove chemical contaminants and finally allowed to settle by gravity. Within the biphasic mixture, the top part consists of the biofuel, whereas the bottom part is glycerol. After decantation and several washings, the composition of biodiesel is analyzed by gas chromatography (Halim et al., 2012; Makareviciene et al., 2013).

The production of fuel starting from a genetically modified algae biomass, capable of going beyond the sustainable production of energy, is the goal of the next generation of biofuels. This could be achieved through new algae strains that are able to capture and store excess CO_2 thus acting as a carbon negative rather than carbon neutral source (Lü et al., 2011; Liew et al., 2014).

Despite many advantages, there are still technological and economic limitations in the production chain of biofuels from algae. The main obstacles are in the selection of algal strains and in the development of an efficient lipid extraction process economically

favorable for production at the industrial scale (Aransiola et al., 2014; Liew et al., 2014).

In particular, the ideal (micro)algal strain for biofuel production should have high lipid productivity, high CO₂ fixation capacity, limited nutrient requirements, a fast growing cycle, high photosynthetic efficiency, outcompete contaminant strains in open pond production systems, be able to produce valuable co-products, and display self-harvesting characteristics (Brennan and Owende, 2013). Currently, there is no (micro)algal strain with all these requirements. Though genetic engineering-inspired modeling will be the tool for success, this remains limited to a few algal laboratory model strains, and additional genome sequencing of strains with appropriate features is required. Microalgae can potentially produce 100 times more oil per acre land as compared to any terrestrial oil-producing crop (www1.eere.energy.gov/biomass; Singh et al., 2011a) which remains, in spite of the fact that the current cost of microalgae per unit mass is higher (Greenwell et al., 2010), an attractive point in terms of lowering fossil fuel dependency.

BIOREFINERY

The generation of a variety of bio-based products next to the production of energy would significantly maximize the value of the used feedstock (Azapagic, 2014). This is achieved taking advantage of various components present in the biomass as well as by adopting different technologies and processes. In particular, microalgae and cyanobacteria show potential since, in addition to biofuel precursors, carbohydrates and cellulose (ideal for the production of fine chemicals), they also produce pigments (e.g., phycocyanine, carotene, astaxanthin) and antioxidants useful in pharmaceutical and cosmeceutical applications, vitamins, and bioactive peptides (e.g., with antihypertensive, anticoagulant, antiviral or antimicrobial activities), and can be very rich in proteins making them suitable for the food and animal feed market (Vanthoor-Koopmans et al., 2012; Jarda et al., 2013; Uggetti et al., in press).

A current trend in the biorefinery is the use of biomass from bacteria and algae associated with wastewater treatment (Olguín, 2012; da Silva et al., 2013; Rawat et al., 2013). The dual purpose of this system makes it one of the most promising strategies in microalgae exploitation owing to the cost-effective and competitive production. Additional studies are required, since there are still limits in the development of an efficient procedure for the separation of the different fractions and for the preservation of the compounds of interest.

EVALUATION OF ENVIRONMENTAL AND SOCIAL IMPACTS OF BIOMASS ENERGY PRODUCTION

Conversion, use and accessibility of energy are basic concepts of sustainability, which need to be coupled to acceptable social, economic, and environmental dimensions. In order to guarantee societal benefits of biofuels production, governments, researchers and companies will need to cooperate in carrying out assessments, mapping suitable areas, defining, and applying national, and international standards, as well as enhancing commercial-scale production (Singh and Olsen, 2012; Rathore et al., 2013).

Lately, a new tool for sustainability assessment of biofuels has been introduced: LCA, which helps policy makers in their choice of the most appropriate biofuels for specific purposes (Clay and Fong, 2013; Kendall and Yuan, 2013).

According to the US Environmental Protection Agency (EPA) a complete LCA process of biofuels includes the evaluation and analysis of every single step of production, from raw material to harvesting, processing, and transport, to their storage and distribution, and to their final use (<http://www.epa.gov/nrmrl/std/lca/lca.html>). The LCA approach has proven a valuable method in understanding the environmental impacts generated by various industrial products during their production, and is now the foremost accepted methodology for the assessment of environmental impacts (e.g., eutrophication, soil erosion, water run-off, loss of natural biota, and land resources) related to the introduction of new-technology fuels (Singh and Olsen, 2012; Ajayebi et al., 2013).

BIOFUELS: CURRENT SITUATION AND NEW SCIENTIFIC RESEARCH TRENDS

The global scenario of “green fuel” production is still imprinted by the presence of first generation biofuels. Main producers of bioethanol are the United States and Brazil and soybean and sugarcane sucrose biomasses are the traditional feedstocks for biodiesel (Koçar and Civaş, 2013; Avinash et al., 2014). However, a trend toward large-scale production of biofuel exists. In Australia the current strategy is to increase the biofuel industry potential by exploiting marginal lands for exotic plant cultivation, while in India many venture assets presently concentrate on the use of invasive and non-edible plants for alternative energy production, even if the use of invasive plants for biofuel is not economical nor sustainable.

Concerning the production of third generation biofuels, strong support comes from scientific research in Europe, China, and the United States, with an emphasis on biomass yield improvements and the exploitation of genetic engineering tools. Several strategies are under investigation to overcome the limitations of large-scale production and to expand the market of this alternative source of energy. In particular, studies on microalgae and plants are aimed to control carbon partitioning during photosynthesis within the cells (Liberton et al., 2013; Melis, 2013). This could result in higher yields and quality improvements of the final products. A recent example is the heterologous expression of the entire isoprene biosynthetic pathway in the cyanobacterium *Synechocystis* PCC 6803 (Bentley et al., 2014).

Another experimental approach aimed at increasing the biomass productivity concerns the RUE enhancement by modulating light capture at the antenna complex. This can be achieved either by optimizing the light distribution within the plant canopy, by extending the PAR spectrum, or by size reduction of the light harvesting antenna (see section Improving Canopy Photosynthetic Performance). Genetic engineering plays a key role in the manipulation of microorganisms, addressing the improvement of their growth rate, production and accumulation of their metabolites and precursors, and harnessing their defenses against microbial competitors, while simplifying harvesting methods (Gimpel et al., 2013; Shurin et al., 2013; Leite

and Hallenbeck, 2014). Bacteria have been engineered to produce a wide range of chemical compounds closely related to fuel molecules, and synthetic biology approaches were applied for the production of advanced biofuel targets (Choi and Lee, 2013; Gronenberg et al., 2013; Wen et al., 2013). Molecular biology also enabled the production of specific yeasts and algae used in biofuel platforms. In particular, engineered yeast cells producing alcohols, sesquiterpenes, and fatty acid ethyl esters have been used for advanced biofuels with improved yield. Furthermore algae have been genetically manipulated to optimize their fatty acid biosynthesis, hence to increase oil content, or to optimize acid chain length for more stable algal biodiesel (Buijs et al., 2013; Gimpel et al., 2013). The CoA carboxylase enzyme, involved in the rate-limiting step in fatty acid biosynthesis, is one of the primary targets for the increase of algal oil yields (Mühlroth et al., 2013). Likewise, to control fatty acid chain length and hence biodiesel quality, thioesterases that terminate the chain elongation in fatty acid biosynthesis and functionally determine the identity of the end product are targeted as well (Blatti et al., 2013).

Research also focuses on the improvement of technologies concerning the collection and the conversion of algal biomass. An efficient harvesting method is a major challenge for the algal biofuel commercialization. Although sedimentation and flocculation seem to be the best low-cost methods they are not suited for all microalgae strains (Vandamme et al., 2013). New methodologies need to be developed and efforts are currently directed toward genetic modification to improve algae collection through the promotion of cell aggregation and the ability to flocculate more easily (Mendez et al., 2009; Scholz et al., 2011).

A recent challenge related to the conversion of algal biomass into biofuel is to achieve a single-step procedure. The development of a “supercritical approach” allowing the direct conversion of wet algae to crude biodiesel is under investigation (Patil et al., 2012; Reddy et al., 2014). It has been shown that supercritical carbon dioxide extracts lipids from algae with more efficiency and higher selectivity than traditional solvent separation methods, while extract purity and the final product concentration remain high. A further improvement would be combining the process of supercritical CO₂ lipid extraction with the use of a suitable solid catalyst to allow extraction and conversion at a single production site (Soh et al., 2014).

The hydrothermal liquefaction for biomass conversion is also an appealing procedure and is particularly suitable for the direct treatment of wet feedstocks (Kruse et al., 2013; Chen et al., 2014; Cheng et al., 2014; Li et al., 2014). This technology allows simultaneous production of value-added compounds and bio-oil from algal biomass while a one-step process for direct liquefaction and conversion of wet algal biomass under supercritical methanol conditions should be possible (Patil et al., 2011).

BIO-BASED POLYMERS

Plastics play a major role in a sustainable development. Being both affordable and highly versatile, they have become essential to meet necessities in sectors such as health, shelter, communication, transportation, and food and energy security. Considering the growing demand for polymer products it is necessary to identify those production procedures which provide the lowest

environmental impact and carbon footprint. While the exploitation of fossil feedstock in the manufacturing of plastics represents a sustainable and effective use of oil and gas, the most successful approach to bio-based polymers is to produce monomers (having at least two reactive groups or one C=C bound) from bio-waste and from “renewable oil” obtained from biorefineries and through plastic degradation. This, in combination with a melt- and gas-phase polymerization process, provides renewable and bio-degradable plastics without impairing their intrinsic characteristics (Mülhaupt, 2013). Examples of bio-based, renewable monomers are ethylene (for poly vinyl chloride or polyethylene glycol) e.g., from bioethanol, diamines (for polyamides and isocyanates) e.g., from amino acids and sugars, phenols (for polycarbonate or polyepoxides) e.g., from lignin, and polyols (for polyurethanes) e.g., from carbohydrates.

SOLAR-ENERGY-CONVERTING TECHNOLOGY MIMICKING NATURAL PHOTOSYNTHESIS

The energy efficiency of natural photosynthesis, defined as the ratio of energy content of the annually harvested biomass versus the annual solar radiation over the same area, rarely reaches 1%, with a theoretical maximum for algal biofuels of 4.5% (Blankenship et al., 2011; Barber and Tran, 2013; Frischmann et al., 2013). As a consequence, biomass-based energy production could provide a limited contribution to our future energy demand. However, the high efficiency of light-harvesting (quantum efficiencies >90%; defined as the probability that an excitation leads to charge separation; Şener et al., 2011) and light-driven water oxidation (>80% in low light conditions; Ananyev and Dismukes, 2005) inspire the development of innovative solar-energy-converting technologies.

PHOTOVOLTAIC CELLS

Photosynthesis consists of a subset of energy conversion processes which can be mimicked in solar energy harvesting devices (Andreiadis et al., 2011). At the most basic level this implies the conversion of sunlight into charge-separated states by which an electron is freed from a light-absorbing semiconductor material (often silicon doped with impurities). The empty space left behind at the site of the electron-emitting atom is often referred to as a “hole.” Photo-generated electrons are conducted toward an acceptor material while holes—which behave as mobile positively charged particles—diffuse into the opposite direction. This separation of hole-electron pairs (called “excitons”) into free electrons and holes is expressed as the photovoltage and is the source of usable electrical energy.

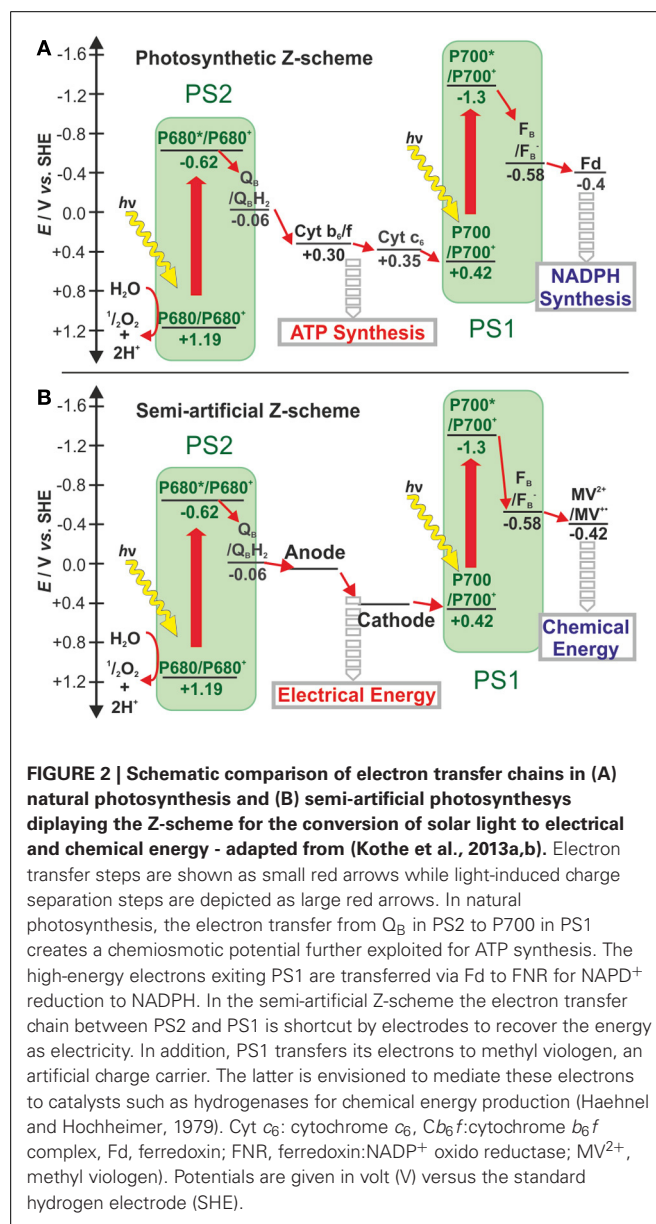
Thus, photovoltaic devices generate electrical energy under illumination by exploiting semiconductor material or molecular photosensitizer for light-induced charge separation. While photovoltaic technology is already implemented at a global scale, the search for cheaper and less energy demanding materials for device fabrication is ongoing. One class of alternative materials for light induced charge separation includes the components of natural photosynthesis, the photosystem protein complexes. Their extreme quantum efficiency and large natural abundances makes them highly suitable for biohybrid photovoltaic devices (Barber and Tran, 2013; Frischmann et al., 2013).

The major consideration is how to avoid charge recombination when photosynthetic biomolecules are integrated in photovoltaic devices since the charge carrier produced should not be quenched at the photoelectrode it originates from but readily transferred to the counter electrode, preferably close to the circuit for effective power generation. A number of complete photovoltaic cells based on photosystem 1 (PS1) and/or photosystem 2 (PS2) are described below.

The first generation of biophotovoltaic cells based on isolated photosynthetic proteins incorporated semi-conducting electron transport layers to interface the biomolecules with the electrodes (Das et al., 2004). A later concept followed dye sensitized solar cell (DSSC) technology. In DSSC, the intrinsic properties of semiconductor materials with high overpotential for specific electron transfer processes are exploited to limit charge recombination processes. The same principles were applied for the construction of a PS1-semiconductor hybrid photoanode (Merishin et al., 2012). The semiconductor properties coupled to the high surface area electrode yielded the highest energy conversion efficiency reported to date for a biophotovoltaic device (0.08%). Still, the performance of the hybrid system remained well below that of state-of-the-art photovoltaic devices. Moreover, biophotovoltaic concepts much rely on the properties of the semiconductor material. The energy-costly fabrication and the light-induced charge separation properties of the semiconductor may thwart the advantage of implementing the biological component. Thus, for photosystem-based hybrids to make any significant impact in sustainability schemes, suitably conductive and cheap electrode materials must be found.

Yehezkeili et al. (2012) reported on a biophotovoltaic cell that fully excluded semiconductors but instead was based on a PS2 photoanode in combination with an oxygen reducing biocathode. Upon charge separation at the PS2 anode, water becomes oxidized and the produced oxygen diffuses to the cathode where it is reduced back to water via an enzyme-catalyzed process. The irreversible electrochemical properties of the charge carrier, oxygen, ensure that its reduction only takes place at the catalyst-modified biocathode. Charge recombination of oxygen at the PS2 photoanode is slow, and short-circuiting is therefore impeded. This concept was recently extended to the combination of a PS2 photoanode with a PS1-based oxygen-reducing photocathode (Kothe et al., 2013a,b; Hartmann et al., 2014). In this approach, oxygen is again exploited as the charge carrier. The additional charge separation step at PS1 allows to couple a catalyst for H_2 evolution or other irreversible reductive processes. This provides the basis for the development of semi-artificial devices that fully mimic the two solar energy conversion steps in natural photosynthesis (Figure 2) (Kothe et al., 2013a,b; Hartmann et al., 2014).

The above illustrates how charge recombination can be avoided by exploiting irreversible charge carriers such as oxygen in the PS2-based devices. However, bringing PS1 in contact to a conductive electrode is more challenging since, in contrast to PS2, PS1 does not catalyze an irreversible chemical reaction but instead exchanges electrons with reversible redox partners. Hence, freely diffusing charge carriers will recombine with each other or with the electrode. The main strategies to minimize such recombination losses rely on (i) contacting both the electron-accepting and



the electron-donating sides of PS1 with surface-confined electron relays or (ii) direct electron transfer between the two electrodes. Here, the challenge resides in the actual cell fabrication since PS1 needs to be inserted into a nanogap between the two electrodes. The underlying concept was shown at the single molecule level in a double junction involving direct electron transfer (Gerster et al., 2012). Although the electric current density and open-circuit voltage of such a bionanodevice opens up prospects for highly efficient biophotovoltaics, upscaling this concept to a large surface area may be difficult since defects in the nanogap would result in direct short-circuits between the two electrodes (Plumeré, 2012). A large-scale device that fully excludes the use of semiconductor material still needs to be demonstrated.

To date, efficiencies of devices that deploy biological components for light-induced charge separation have not yet exceeded 0.1%, which is well below those seen for standard semiconductor

photovoltaics. A further improvement in performance requires alternative strategies to circumvent charge recombination, in particular for PS1-based devices (Kothe et al., 2014). One approach that has been overlooked so far is to mimic the role of NADPH in nature i.e., the role of O₂ in the PS2-based system, namely to develop for PS1 an electrochemically irreversible charge carrier that requires a catalyst for electron transfer. Also, an enhanced loading of photosynthetic protein may eventually result in higher photocurrent densities. When the electronic communication between the photosynthetic protein and the electrode is based on a monolayer design, porous high-surface-area electrodes may be used. However, this would imply the use of transparent materials therefore rely on expensive semiconductors. Instead, the technological trend may move from monolayer layouts toward multilayers that are contacted via conducting polymeric matrices. In this case, transparency and electron transfer properties within the polymer-protein hybrid film must be further developed to realize highly efficient biophotovoltaic systems. Moreover, in the development of high performance biophotovoltaics, the stability under illumination is also a critical factor. In this regard, since PS1 is more stable as compared to PS2, the former may yet be better suited for the development of energy conversion devices.

FULLY-INTEGRATED ARTIFICIAL PHOTOSYNTHETIC SYSTEMS

The term artificial photosynthesis was coined for the first time to make a distinction between conventional silicon-based and novel dye-sensitized solar cells (DSSC) (Grätzel, 1991). In contrast to the direct electron transfers from the silicon atoms upon light absorption, the DSSC separates light absorption by “sensitized” metal-complexed or organic metal-free dye molecules which subsequently inject electrons into semiconducting material. This way, energy conversion efficiencies of up to 10% can be obtained (Nazeeruddin et al., 1993). Although the original DSSC necessitates a liquid electrolyte, affecting device stability, recent advances have shown the possibility for these solar cells to perform well as solid state devices using hybrid perovskite dyes (Burschka et al., 2013). Perovskite-based technologies and architectures for solar cells are constantly being improved and they show great promise for the realization of artificial photosynthesis (Zhou et al., 2013; Christians et al., 2014).

The success of the DSSC in terms of efficiency and cost effectiveness has been an important driver for artificial photosynthesis research targeting the more complex energy conversion processes of natural photosynthesis. A major target to mimic are the reactions that involve the splitting of water. While in natural photosynthesis the protons form a gradient across the cell membrane to drive ATP synthesis, energy conversion devices rely on the subsequent formation of molecular hydrogen as a combustible fuel. In the realm of semiconducting materials, water splitting is achieved by means of solar cells that generate high photovoltages. In a hybrid technology the semiconducting material is interconnected with catalytic material to enhance the process. Concomitantly, catalytic material is being developed to replace the platinum, a highly precious metal, generally employed for hydrogen formation. Significant progress has been made especially for artificial water-splitting by the discovery of a cobalt-based catalyst that can be incorporated into energy conversion devices. In the past few

years fully integrated devices operating on these principles have been reported (Reece et al., 2011; Zhao et al., 2012; Abdi et al., 2013).

In spite of a promising 10% energy conversion efficiency—the minimal standard to make an impact at an industrial scale—for some of these devices, they are not yet commercially competitive with fossil fuels and in the past two decades DSSC efficiency could not be significantly improved. The search is therefore for “dirt cheap” materials, available at low fabrication costs but available in great abundance. Chemically synthesized supramolecular systems can act as homogeneous devices that simultaneously enable light absorption, electron transfer and catalysis. Promising novel materials are dyads, triads, or more complex supermolecules (Megiatto et al., 2012) but they have not yet been integrated within a photovoltaic device. Other potentially very cheap molecular systems consist of photovoltaic solar cells made of conducting polymers doped with dye molecules (Liang et al., 2010). Like all molecule-based solar cells, such devices need to address stability issues under long term operation and resilience toward air (Brabec et al., 2010).

Possibly the cheapest material imaginable is the actual photosynthetic machinery found in photosynthetic organisms. At the level of proteins and protein complexes, the catalytic material is ready-made with proven functionality, consists of ubiquitous substances, and is powered by solar energy while assembled by utilizing carbon dioxide. Most research to date focuses on the interconnection of protein complexes with conducting materials. Some fully integrated photovoltaic devices may serve as proof of principle despite very low efficiencies (Das et al., 2004; Mershin et al., 2012). The main target for artificial photosynthesis beyond photovoltaics is the implementation of PS 1 complexes, generating high energy electrons. In conjunction with platinum electrodes or nanoparticles, photogenerated electrons can be employed for hydrogen generation (Grimme et al., 2008; Iwuchukwu et al., 2009). Complemented with PS 2 and a hydrogen evolving enzyme, a complete biohybrid device can be envisioned (Badura et al., 2006). To date only partially integrated systems, consisting of PS1 complexes fused with hydrogenase for homogeneous catalysis (Ihara et al., 2006) or photoelectrochemical catalysis on gold (Krassen et al., 2009) have been reported.

An additional development is the capture of carbon dioxide and its photoelectrochemical conversion to carbonhydrogen molecules (Spinner et al., 2011). Ideally this conversion reaction would yield a gas or fluid fuel that might be easier to handle than hydrogen gas. Future devices incorporating such reactions will likely compare to those currently used for bulk chemical synthesis, allowing the use of existing factories, and hence they could rapidly become cost effective.

IMPLICATIONS OF OXYGENIC PHOTOSYNTHESIS FOR HUMAN SPACE EXPLORATION AGRICULTURE IN SPACE

Photoautotrophic organisms such as green plants and alga, and cyanobacteria, are essential to support human life in long-term stationary or interplanetary missions because they scrub the crew's air of carbon dioxide by atmospheric fixation, produce oxygen, adjust humidity or recycle wastewater, and convert

organic wastes back into edible mass. Physical factors in space that might affect photosynthesis and carbon utilization are solar and cosmic radiation, gravity, temperature, hypobaria, humidity, light, and the absence of an Earth magnetic field (i.e., at the Moon and at Mars). Because earth organisms are evolutionary ill prepared for microgravity (10^{-6} – 10^{-3} g), fractional gravity (0.17 g for the Moon, 0.38 g for Mars), or cosmic radiation, much space research on photoautotrophs has focused on the biochemical or physiological effects of real or simulated low gravity and ionizing radiation. For agriculture in space the deployment of microalgae may be particularly rewarding as some are very nutritious and can be directly consumed with little processing. They also respond much faster to environmental changes and hence are easier to investigate. Moreover, microalgae are generally robust organisms with highly efficient photosynthesis and are ideal organisms for life support systems (LSS) as they can grow in panel- or tube-fitted reactors within limited confinements (see further and review by Saei et al., 2013).

Microgravity and ionizing radiation: physical constraints of sustained life in space?

Because of the constant presence of a gravity vector on Earth, plants have learned to use this force for many biological functions and mechanisms. Hence, the loss or change of gravity alters the way in which plants sense and respond to environmental cues. For instance, *Arabidopsis* hypocotyls growing in microgravity at the ISS display a novel phototropic response to red light, which is suppressed at 1-g simulated gravity using an on-board centrifuge and in ground controls (Millar et al., 2010). Likewise, seedlings pre-treated with red light experienced a more pronounced blue-light-induced phototropism in microgravity as compared to 1-g controls. Later studies at the ISS confirmed these effects and showed that 0.1–0.3 g sufficed to attenuate the red-light-based phototropism while at a gravity of 0.3 g or above, blue-light phototropism was no longer enhanced. Clearly, conditions at the ISS provide a unique environment to address fundamental questions in space biology (Olsson-Francis and Cockell, 2010; Paul et al., 2013). Importantly, plants grown in the reduced gravities of the Moon (0.17 g) and Mars (0.38 g) are not necessarily exposed to gravity-related stress and so might function normally as if they were on 1-g Earth (Kiss, 2014). Still, more studies with more plants are needed to define the exact threshold for gravity as a prerequisite to (normal) plant life.

In terms of radiation, total dose rates¹ within Low Earth Orbit spacecraft (somewhere between 160 and 2000 km above the Earth's surface) average from $150 \mu\text{Gy d}^{-1}$ to $500 \mu\text{Gy d}^{-1}$ (Benton and Benton, 2001; Goossens et al., 2006; Vanhavere et al., 2008) as compared to the Earth background radiation levels of circa $1\text{--}3 \mu\text{Gy d}^{-1}$ at sea level. During the Apollo missions, with lunar surface exploration lasting 21–75 h and full passage through the Van Allen belts, but no enhanced solar activity, dose rates measured between 180 and $1270 \mu\text{Gy d}^{-1}$ (Bailey, 1975). Dose rates at the lunar surface were measured between 200 and

$360 \mu\text{Gy d}^{-1}$. Comparably, based on ~ 300 days of observation by the Mars Curiosity Rover science laboratory (MSL), the daily space radiation dose at Mars' surface averaged at $210 \mu\text{Gy d}^{-1}$ which was enhanced by roughly 30% during the Solar Particle Event (SPE) of 11 and 12 April 2013 (Hassler et al., 2014).

The survivability after irradiation has been documented for some organisms in terms of the acute lethal dose (LD_{50} in parentheses): humans (4 Gy), mice (4.5 Gy), chicken (10 Gy), fruit fly (640 Gy), onion (20 Gy), wheat (43 Gy), potato (120 Gy), tomato (150 Gy), amoeba (1 kGy), tardigrades (5 kGy), algae (60 Gy– 1.2 kGy^2), and bacteria (60 Gy–30 kGy) (see also <http://www.unscear.org>). A few cyanobacteria (*Anabaena torulosa*, 5 kGy, (Singh et al., 2010); *Croococcidiopsis* sp. 029, 15 kGy, (Billi et al., 2000) and algae [*Coccomyxa actinabiotis*, 20 kGy, (Rivasseau et al., 2013)] have been reported as being highly resistant against radiation although the underlying mechanisms have not yet been studied. While it is reassuring that yearly radiation dose rates in space or on the Moon or on Mars will remain below 1 Gy so that growth of plants and microalgae or cyanobacteria will not be impaired, it is obvious that such dose rates may cause DNA mutations with potential detrimental effects. Hence, extensive research on the effects of cosmic radiation on these organisms, in particular for chronic exposures, is fully warranted.

A brief history on space biology of plants (and microalgae/cyanobacteria)

Plant research in space started in 1971 with a tiny greenhouse called Oasis, at Salyut 1. After many setbacks in the following decade, *Arabidopsis* grown on Salyut 7 finally produced viable seeds and wheat and mustard were later successfully grown (Ivanova et al., 1993; Mashinsky et al., 1994; Salisbury and Clark, 1995). Meanwhile, at the Spacelab module, a European-American venture, unaware of the earlier Soviet results, encountered (and overcame in 1996) the same developmental problems of space-grown plants (Freeman, 1998; Ivanova et al., 1998). The main culprit appeared to be ethylene, which acts as a hormone and is produced by most plant organs (Chaves and Mello-Farias, 2006). On Earth, ethylene is dispersed by air movement but not so in microgravity where it surrounds the plant, enhancing withering and promoting male sterility. Hence, space greenhouses were fitted with fans and ethylene filters and equipped with humidity, CO_2 , temperature, and oxygen sensors so that the seed-to-seed cycle in space could be finally realized (reviewed by Casado, 2006).

Plants have also been studied when exposed to the open space environment in the EXPOSE-E missions (Rabbow et al., 2012) outside the ISS. At low shielding, an average dose rate of $400 \mu\text{Gy d}^{-1}$ was measured, with a total exposure dose of 215 mGy at the lowest shielding (Berger et al., 2012). Remarkably, seeds of *A. thaliana* and *Nicotiana tabacum* (tobacco) could still germinate after 1.5 years exposure—which also included solar UV, cosmic radiation, temperature fluctuations, and space vacuum (Tepfer et al., 2012). During the same EXPOSE-E mission, it was shown that some cyanobacteria and green algae survived the same length of direct exposure (Cockell et al., 2011; Onofri et al., 2012).

¹Dose rates represent the amount of energy deposited per unit mass, with 1 Gy being 1 J/kg. Health risks are generally expressed in mSv/yr, applying organ-specific and radiation type-specific quality factors.

²IAEA Technical Report series 172, Vienna, Austria, 1976.

Technological advances for “green” research in space

Novel lighting technologies for space green houses make use of light-emitting-diodes (LEDs) which are more durable and reliable than the conventional light sources and allow to simulate parts of the spectrum not present in traditional lighting. They can also emit photons within very narrow bands of the spectrum, i.e., only wavelengths needed by the photosynthetic organisms, hence saving energy. The growth of plants (or microalgae/cyanobacteria) on narrow bands of the spectrum is very useful because some chlorophylls use mostly blue and red wavelengths. For instance, red LEDs were used as a photon source in the Astroculture3 growth chamber (Massa et al., 2006).

Advanced methods of soil-free plant growth are crucial to the future of agriculture in space. Such hydroponic and aeroponic systems (<http://en.wikipedia.org/wiki/Hydroponics>) require less water, allow recycling and better uptake of nutrients, can be better controlled, and can be stacked in confined spaces (“vertical farming”). Although they are energy-demanding as they incorporate lighting, pumping, and air moderation, they may form the key for the long-term colonization of Mars and the moon where nutritious perlite-like minerals could be extracted from local soils and where energy production from solar or nuclear energy is not an important issue. In addition, hydroponic and aeroponic methods can be integrated into bioregenerative life support systems (Paradiso et al., 2014)—see further.

Another important aspect is improved greenhouse equipment. For instance, the Commercial Plant Biotechnology Facility (CPBF; www.nasa.gov) integrates Astroculture™ technologies, state-of-the-art control software, fault tolerance and recovery technologies, and telescience capability. It also includes LED technology (red and blue at 670 and 450 nm, respectively) and a Fluorescent Light Module (FLM) using high output bi-axial tubes to ensure maximum photon flux. Lately, attention is being directed toward plant growth under fractional gravity in a prelude toward space biology in reduced gravities of the Moon and Mars. In 2015, NASA will attempt to grow arabidopsis, basil, sunflowers, and turnips on the Moon (0.17 g gravity) in small aluminum cylinders brought to the Moon by a robotic spacecraft, the Moon Express lander. Later, similar small greenhouses will be tested at Mars.

Today, “-omics” developments have proliferated the amount of genomic information to a staggering level. Since the *Arabidopsis Genome Initiative* in 2000, many more plant genome sequences were obtained, including for instance those of C4 crops sorghum, maize, and millet, and the C3 crops wheat, barley, rice, soybean, flax, mustard, cucumber, tomato, potato, banana, peach, pear, apple, orange, and wild strawberry. The genome sequencing of microbes gained equal pace with currently around 2500 bacterial and 150 eukaryal genomes fully sequenced, including 71 cyanobacteria and 7 green algae (<http://genomesonline.org/>). More cyanobacterial and algal genome projects are planned or on-going. For plants, genome projects exist for peanut, chestnut, lettuce, sugar beet, water melon, and coffee. This sequence information can be put to use for the genetic modification of space-dedicated crops or biosphere-supporting bacteria, while genetic responses induced by space conditions will be better understood.

LIFE-REGENERATIVE SUPPORTING SYSTEMS

A person in space needs at least 22.4 kg of food, water, and oxygen per day (Nelson et al., 2003). For a crew of three astronauts flying to Mars and back, with the fastest return currently achievable i.e., without landing on the Martian surface, an estimated 34 tonnes of supplies would be required. It would be impossible to launch and carry this bulk of weight from Earth. Hence material recycling is the only solution and successful food production in space is thus essential. Microalgae, next to plants, claim a prominent role in food supply routes of Moon or Mars stations but they particularly come into play in spacecraft where weight and volume are critical issues (Hendrickx and Mergeay, 2007; Tikhomirov et al., 2007). The green alga *Chlorella* and the multicellular cyanobacterium *Arthrospira* (aka *Spirulina*) already have proven their nutritious value and are now globally commercialized—for full review see Chacón-Lee and González-Mariño (2010).

The purpose of Life Support Systems (LSS) is to transform waste and regenerate air, water, and food. Whereas physico-chemical LSS are based on filtration rounds, chemical treatments, and transition processes, bioregenerative LSS (BLSS) use microorganisms and/or plants for oxygen production and carbon dioxide reduction. The main experimental BLSS that use plants and microalgae and that recycle products in a closed loop are summarized in **Table 1**.

Perhaps the currently best studied system is MELISSA (Micro Ecological Life Support System Alternative) funded by the European Space Agency (ESA). It consists of four interconnected biological compartments and is completely reliant on photosynthesis. Food and oxygen are produced in the photosynthetic compartment consisting of a plant chamber (IVa) and a microalgae cultivation unit (IVb) (**Figure 3**). Preferred plant species are those that provide protein-rich food with high nutritious value, such as wheat, soybean, and potato, next to other crops like rice, tomato, lettuce, spinach, and onion (Paradiso et al., 2014). For IVb the cyanobacterium *Arthrospira* sp. PCC 8005 was chosen as *Arthrospira* species are efficient photoautotrophs that produce

Table 1 | The main experimental BLSS that use plants and microalgae and that recycle products in a closed loop.

System	Country	Period	Microorganism	References
BIOS	Russia	1960	<i>Chlorella</i>	Gitelson et al., 1989
CEBAS ^a	Germany	1985	<i>Chlamydomonas</i>	Blüm, 1992; Blüm et al., 2003
MELISSA ^b	Bel, Fra, Spa, Can	1988	<i>Arthrospira</i>	Mergeay et al., 1988; Hendrickx et al., 2006; Lasseur et al., 2011
CERAS ^a	Japan	1997	<i>Euglena</i> , <i>Chlorella</i> , <i>Arthrospira</i>	Takeuchi, 1997; Omori et al., 2001; Takeuchi and Endo, 2004
CAES ^a	China	2004	<i>Chlorella</i>	Wang et al., 2007

^a contains also an aquatic animal habitat.

^b <http://ecls.esa.int/ecls/>

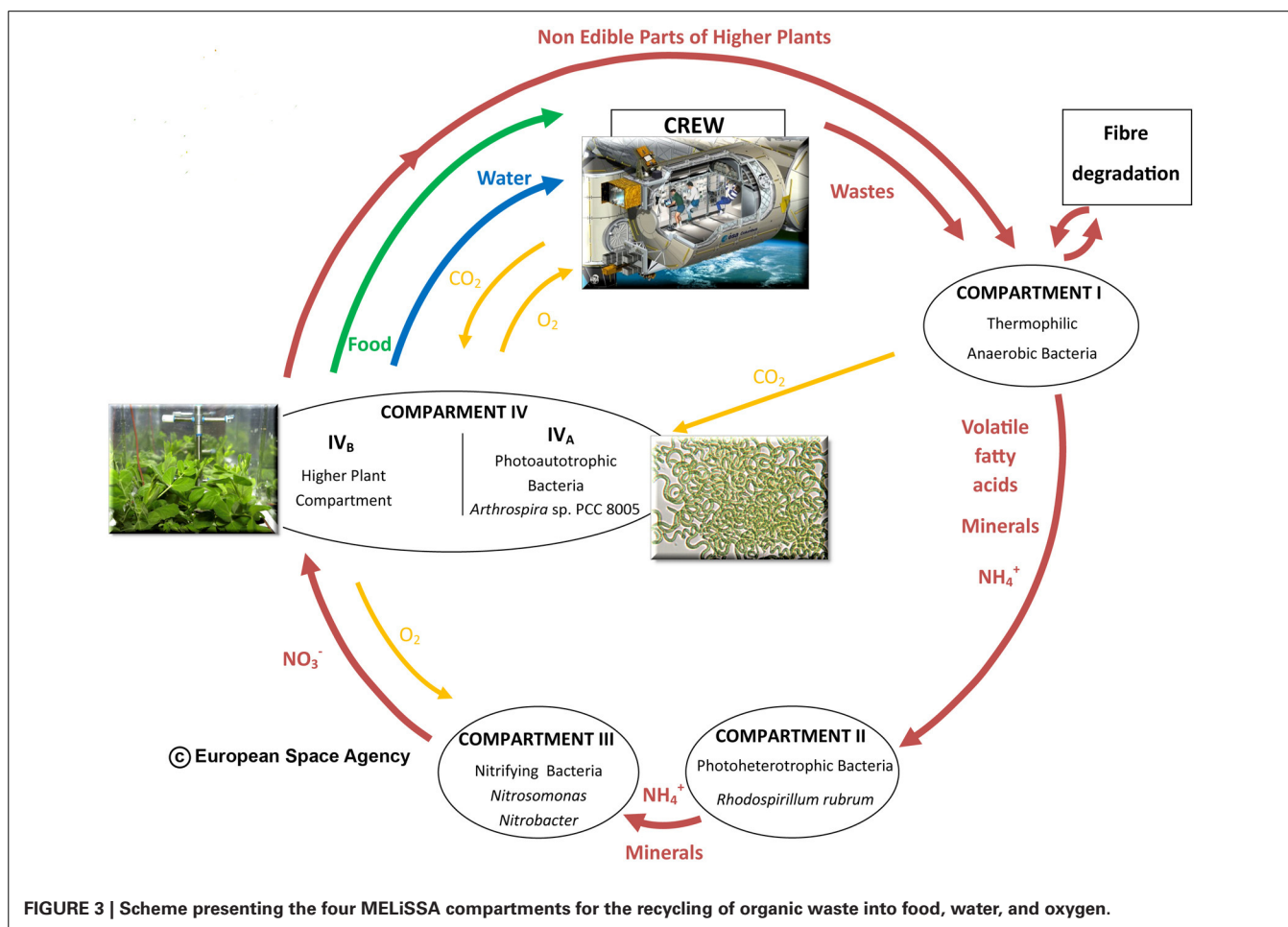


FIGURE 3 | Scheme presenting the four MELiSSA compartments for the recycling of organic waste into food, water, and oxygen.

high amounts of digestible proteins (50–70% of biomass), beta-carotene, omega-6 fatty acids, phycocyanin, important minerals, and antioxidants (Koru, 2012). Marked activities of *Arthrospira* on human health include immunomodulation and anticancer, antiviral, and antibacterial activities while it has positive effects against inflammatory allergy, anemia, cytotoxicity, and radiation sickness (for full review see Ravi et al., 2010 and Sotiroudis and Sotiroudis, 2013).

It was recently shown that acute gamma radiation doses of up to 1600 Gy did not affect the photosynthetic capacity of *Arthrospira* as measured by PSII quantum yield and filaments proliferated normally (H. Badri, pers. comm.). This unexpected radiotolerance is reassuring in view of the relatively high radiation dose rates in Space (0.2–1.2 mGy d⁻¹). However, the possibility of genetic changes induced in *Arthrospira* by chronic exposure to cosmic radiation cannot be excluded. The photosynthetic *R. rubrum* (which is in principle edible as well although it is not considered a primary food source in the MELiSSA concept) has previously been studied under real and simulated cosmic radiation and microgravity (Mastroleo et al., 2009). Similar work is now planned by the ESA for *Arthrospira* sp. PCC 8005 since its 2010 genome data (Janssen et al., 2010) have been very recently updated in an assembly of six ordered genome segments and a unique tiling microarray for all its genes has been constructed (P. Janssen, unpublished).

Clearly, bioregenerative life support systems such as MELiSSA show much promise, in particular when very high recycling efficiencies close to 100% can be achieved and taking into account that very few moving parts are used so that minimal maintenance is expected. The selection of the most hardy and most useful photosynthesizers, whether natural, purposely bred, or transgenic, is a challenge that can be met (Lehto et al., 2006; Saei et al., 2013). The only possible drawback of biological systems is that they are dynamic and that genetic changes induced by space conditions may lead to diminished functionality of the reactor loop. Hence, important issues for BLSS to be addressed in the near future are strict quality control, early warning mechanisms, guidelines for counter measures, and ultimately the ability of self-repair.

CONCLUSIONS

Space biology underlines the great potential that exists in the exploitation of the photosynthetic system since this addresses various aspects to produce food, including the recycling of materials and waste management for sustainability of life. Also, technological advances in green space research including lighting, hydro- and aeroponic plant growth, vertical farming, and microbial purification of gray water are generally applicable to improve living conditions on earth. However, in order for such novel applications to make an impact on society, proper technology and knowledge transfer must be guaranteed.

In addition, it will be important to address not just the quantitative aspect of production (e.g., higher yields) but also the qualitative side, sustaining a bio-based economy by also growing minor crops in environmental niches, the efficient use of resources, and an adequate input control. In the present era of climatic, economical, and societal changes, a better understanding of such a fundamental process as photosynthesis is expected to bring about important new technological and scientific initiatives.

Ideally, knowledge acquisition should drive sustainable, science-based innovation. In this, scientists must face their responsibility by communicating to the wider public that, for the development of a sustainable life, much can be learned and achieved by the keen observation of natural processes.

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Genus *Cistus*: a model for exploring labdane-type diterpenes' biosynthesis and a natural source of high value products with biological, aromatic, and pharmacological properties

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The family Cistaceae (Angiosperm, Malvales) consists of 8 genera and 180 species, with 5 genera native to the Mediterranean area (*Cistus*, *Fumara*, *Halimium*, *Helianthemum*, and *Tuberaria*). Traditionally, a number of *Cistus* species have been used in Mediterranean folk medicine as herbal tea infusions for healing digestive problems and colds, as extracts for the treatment of diseases, and as fragrances. The resin, ladano, secreted by the glandular trichomes of certain *Cistus* species contains a number of phytochemicals with antioxidant, antibacterial, antifungal, and anticancer properties. Furthermore, total leaf aqueous extracts possess anti-influenza virus activity. All these properties have been attributed to phytochemicals such as terpenoids, including diterpenes, labdane-type diterpenes and clerodanes, phenylpropanoids, including flavonoids and ellagitannins, several groups of alkaloids and other types of secondary metabolites. In the past 20 years, research on *Cistus* involved chemical, biological and phylogenetic analyses but recent investigations have involved genomic and molecular approaches. Our lab is exploring the biosynthetic machinery that generates terpenoids and phenylpropanoids, with a goal to harness their numerous properties that have applications in the pharmaceutical, chemical and aromatic industries. This review focuses on the systematics, botanical characteristics, geographic distribution, chemical analyses, biological function and biosynthesis of major compounds, as well as genomic analyses and biotechnological approaches of the main *Cistus* species found in the Mediterranean basin, namely *C. albidus*, *C. creticus*, *C. crispus*, *C. parviflorus*, *C. monspeliensis*, *C. populifolius*, *C. salviifolius*, *C. ladanifer*, *C. laurifolius*, and *C. clusii*.

Keywords: *Cistus*, biosynthesis, labdane-type diterpenes, phenylpropanoids, biological action, genomic approaches

INTRODUCTION

Cistus L. (from the Greek word kistos-κίστος) or rock rose, is a genus of dicotyledonous perennial herbaceous plants that have hard leaves and grow in open areas of stony and infertile soils. They are indigenous to the Mediterranean region and are known for their durability. Even after natural regional forest fires, these plants are capable to grow due to their increased seed germinability after exposure of the seeds to high temperatures (Thanos et al., 1992). In some species seasonal dimorphism is observed, enabling the plants' adaptation to drought conditions, which induces leaves to decrease in size and grow more hair (Aronne and Micco, 2001). The characteristic feature of the genus is a combination of diverse hair types on the leaf, stem, and calyx including non-glandular trichomes. The tufted and stellate as well as the elongate glandular trichomes produce and

secrete a resin. In some species (e.g., *C. creticus* subsp. *creticus*), this resin is rich in biologically-active and pharmacologically-interesting metabolites, such as flavonoid aglycones, glycosides, and terpenoids including labdane-type diterpenes. Since the original description of the genus in 1753 by Linnaeus, a number of species and subspecies have been categorized within *Cistus*. After several taxonomic re-evaluations, about 21 species of *Cistus* are now recognized spreading within the white and pink-flowered lineages (Guzmán and Vargas, 2005). *Cistus* species are distributed both in the eastern and western Mediterranean, where the highest diversity is observed, and are also widespread in the Balearic and Canarian islands (Guzmán and Vargas, 2005, 2010). Several of them have been employed in Mediterranean folk medicine as herbal tea infusions for healing digestive problems and colds, as extracts for the treatment of diseases, and as

fragnances. The resin, *ladano*, produced by *C. creticus* in Crete, Greece and Cyprus, and *C. ladanifer* in Spain, is exported to a number of Arabic countries where it is used as incense.

In the past 20 years, research on *Cistus* was of chemical, biological, and phylogenetic nature. Added to this list are the recent genomic and molecular studies. Metabolomic analyses using chromatographic and spectroscopic tools allowed the identification of several chemical groups with distinct biological activities. Among the most important compounds are terpenoids, including diterpenes, labdane-type diterpenes and clerodanes, phenylpropanoids, including flavonoids and ellagitannins, several groups of alkaloids and some other secondary metabolites.

A plentiful of biological functions have been attributed to the resin produced by these species. Pharmacological studies on *Cistus* extracts have demonstrated their action as antioxidants (Attaguile et al., 2000; Hernández et al., 2004; Sadhu et al., 2006; Sarić et al., 2009; Amensour et al., 2010; Barrajón-Catalán et al., 2010; Akkol et al., 2012; Riehle et al., 2013; Zidane et al., 2013), antibacterial and antifungal (Chinou et al., 1994; Bouamama et al., 1999; Barrajón-Catalán et al., 2010; Barros et al., 2013), antiviral (Droebner et al., 2007; Ehrhardt et al., 2007), anti-cancer (Chinou et al., 1994; Demetzos et al., 1994a, 2001; Dimas et al., 1998; Angelopoulou et al., 2001a; Dimas et al., 2006; Hatziantoniou et al., 2006; Barrajón-Catalán et al., 2010; Skorić et al., 2012), and other functions discussed later in the review.

The ability of several *Cistus* species to produce high amounts of natural metabolites makes them attractive models for the elucidation of their biosynthetic pathways. The pathway leading to the production of terpenes, especially labdane-type diterpenes, has been investigated in *C. creticus* subsp. *creticus* and several genes have been characterized (Falara et al., 2008, 2010; Pateraki and Kanellis, 2008, 2010). Among these are the germacrene B synthase (*CcGrB*), (Falara et al., 2008), the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (*CcHMGR*), DXP reductoisomerase (*CcDXR*) and 1-deoxy-D-xylulose-5-phosphate synthase (*CcDXS*) (Pateraki and Kanellis, 2010), two active homologs of geranyl-geranyl diphosphate synthase (*CcGGDPS1*, *CcGGDPS2*) (Pateraki and Kanellis, 2008), and copal-8-ol diphosphate diterpene synthase (Falara et al., 2010).

In this review we focus on the major representatives of *Cistus* species: *C. albidus*, *C. creticus*, *C. crispus*, *C. parviflorus*, *C. monspeliensis*, *C. populifolius*, *C. salviifolius*, *C. ladanifer*, *C. laurifolius*, and *C. clusii*, which are commonly found in the Mediterranean basin. Due to their plethora of uses and potential valuable therapeutic activities they have been extensively studied.

SYSTEMATICS OF *CISTUS* SPECIES

The family Cistaceae (Angiosperm, Malvales) consists of 8 genera (Arrington and Kubitzki, 2003) and 180 species, with 5 genera native to the Mediterranean area (*Cistus*, *Fumara*, *Halimium*, *Helianthemum*, and *Tuberaria*). The taxonomic separation of the genus is based on phenotypic observations, including morphological characters like shape, nerve number, color and trichomes of leaves and stems, and reproductive characteristics such as petal and sepal number, shape and color of flowers, number of fruit valves and style size. The phenotype-based genus taxonomy

was confirmed recently using plant chemotype and molecular approaches.

Taxonomic classification of *Cistus* was formed prior to 1800 (Linnaeus, 1753), but the first integrated separation was implemented in 1824 by Dunal (1824), who described 28 species divided in 2 sections, *Erythrocistus* and *Ledonia*. Shortly thereafter, Sweet (1830) described 33 species, also divided into *Erythrocistus* and *Ledonia*, where 3 additional species in section *Erythrocistus* and 7 species in section *Ledonia* were included. Spach (1836) separated them in 5 genera, named *Ladanium*, *Rhodocistus*, *Stephanocarpus*, *Ledonia* and *Cistus*, further divided into sections *Rhodopsis*, *Eucistus*, and *Ledonella*. The plant species divided in subgenera *Erythrocistus* and *Ledonia* were further separated into 7 sections: *Macrostyli*, *Brachystyli*, and *Astyli* in subgenus *Erythrocistus* and *Stephanocarpus*, *Ledonia*, *Ladanium*, and *Halimioides* in subgenus *Leucocistus* (Willkomm, 1856). Grosser (1903) described 3 groups distributed into 16 species in 7 sections: Group A contained *Rhodocistus*, *Eucistus*, and *Ledonella* while Groups B and C, respectively, made up of *Stephanocarpus* and *Ledonia*, and *Ladanium* and *Halimioides*. Dansereau (1939) classified the species in subgenera *Erythrocistus* and *Ledonia*, like Willkomm, and then separated them in 8 sections, with naming *Macrostyli*, *Erythrocistus*, and *Ledonella* for sections of subgenus *Erythrocistus*, and *Stephanocarpoidea*, *Stephanocarpus*, *Ledonia*, *Ladanium*, and *Halimioides* for sections of subgenus *Leucocistus*.

More recently, Demoly and Montserrat (1993) described the distribution of 12 species of genus *Cistus* that grow in Iberia. In this approach, 3 subgenera were classified: I. subgenus *Cistus*, containing *C. albidus*, *C. creticus*, *C. crispus*, and *C. heterophyllus*; II. subgenus *Leucocistus*, containing *Ledonia* with species *C. monspeliensis*, *C. salviifolius*, *C. psilosepalus*, and *C. populifolius*, and section *Ladanium* with *C. ladanifer* and *C. laurifolius*; and III. subgenus *Halimioides* containing *C. clusii* and *C. libanotis*. From this study, it became apparent that most *Cistus* species grow in western Mediterranean. The same conclusion was reached for the species distribution (Table S1), where the main 10 *Cistus* species, discussed in this review, are distributed in 28 areas. Specifically, numerous species grow in Spain followed by Morocco, Italy, Portugal, Algeria, and France. Conversely, in the eastern Mediterranean the number of species is low with the most widespread species being *C. creticus*, *C. palviflorus*, and *C. salviifolius*.

A recent classification of *Cistaceae* is based on combined nuclear (ncpGS, ITS) and plastidic (*trnL-trnF*, *trnK-matK*, *trnS-trnG*, *rbcL*) DNA sequence comparisons, which divided *Cistus* into 3 subgenera (similar to Demoly and Montserrat, 1993): the purple flowered subgenus *Cistus* and the white flowered subgenera *Leucocistus* and *Halimioides* (Figure 1) (Guzmán and Vargas, 2005; Guzmán et al., 2009). Interestingly, *C. palviflorus* appeared most closely related to subgenus *Leucocistus* (white flowers), although it possesses light purple flowers. Similar observations led in the past to the creation of a separate section for *C. palviflorus*, namely *Ledonella*. In another work, the evolution of family Cistaceae was studied by the phylogenetic analysis of plastid *rbcL* and *trnL-trnF* sequences (Guzmán and Vargas, 2009). This study confirmed the clear separation of the genus into 2 groups, with purple (excluding *C. palviflorus*) and white flowers

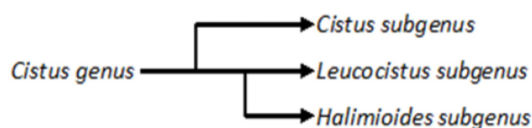


FIGURE 1 | Classification of three subgenus of *Cistus* genus based on analysis of *trnF*, *matK* and ITS sequences (Guzmán and Vargas, 2005) and plastid *rbcl* and *trnL-trnF* sequences (Guzmán and Vargas, 2009).

and certified the family Cistaceae as monophyletic, sisterly to families Dipterocarpaceae and Sarcolaenaceae (Guzmán et al., 2009). A similar classification was achieved by analyzing polyphenolic composition of aerial parts of the most common species, which separated *Cistus* subgenus from the two other subgenera by its higher flavonoid content (Barrajón-Catalán et al., 2011).

Another phylogenetic study confirmed the chemical and genetic (ISSR—PCR amplification) differentiation between the *C. creticus* subspecies *eriocephalus* and *corsicus* (Paolini et al., 2009). The affinity and distance estimation between individual plants of *C. creticus* L. in Corsica and Sardinia, as inferred by the *trn-F* and *RPL32-TRNL* sequences of *cpDNA*, showed that the plants were divided into 4 groups having evident correlation with the region (Falchi et al., 2009). In another taxonomic approach the composition of essential oils of *C. salvifolius* in 15 Cretan (Greece) populations was studied, dividing the plants into 3 groups, most of them belonging to the group with high camphor production (Demetzos et al., 2002b). Also, a chemometric interpopulation study of *C. creticus* in the East and West parts of Crete showed the existence of a high variability within the essential oils. The plants were geographically divided into three groups, two in the West and one in the Eastern part of Crete (Demetzos et al., 2002a).

BOTANICAL CHARACTERISTICS

GENUS *CISTUS* L

Cistus plants are small, woody shrubs with a straight stem that has opposite rich-spreading branches and can reach an average of one meter in height (Sweet, 1830). On branches grow usually corrugated leaves, simple and indivisible, either petiolate or sessile, growing opposite or alternate, and carry simple epidermal hair (trichomes) either asteroid or in bunches. Two types of trichomes appear in *Cistus*, the non-glandular or stellate and the glandular which secrete a resinous exudate, the ladano, to which they owe their distinctive aromatic scent (Gulz et al., 1996). The plants have a terminal or axillary cymose inflorescence, in some species racemose or umbel, with unilateral scorpioid cyme, and by reduction solitary flowers (Demoly and Montserrat, 1993). The flowers are ephemeral, stimulated by the morning light, have either white or pink/purple petals and 3–5 petals. There are numerous stamens, while the ovary has 5 carpels (but can also be 6–12), the style is straight, usually long and inconspicuous, and the stigma is large, discoid with 5–12 lobes (Demoly and Montserrat, 1993). *Cistus* plants have numerous polyhedral seeds with two linear cotyledons. Their chromosome number is $n = 9$ ($2n = 18$) (Demoly and Montserrat, 1993). The plants form

ectomycorrhizal roots through strict symbiotic associations with various mycorrhizal fungal species, mainly belonging to genus *Lactarius* (Comandini et al., 2006). These are characterized by a multilayered mantle and a Hartig net hyphae physiology that involves both epidermal and cortical cell layers (Comandini and Rinaldi, 2008).

The specific botanical characteristics such as are color of petals, number of sepals, and fruit compartments, type of leaf base and size of the styles are the most commonly used in *Cistus* systematic classification (Table S2).

Their general morphological characters as well as their adaptability mechanisms to various harsh environmental conditions will be briefly discussed.

Subgenus I: *Cistus* L

Flower morphology is characteristic in this subgenus. Specifically, each flower consists of five sepals, with pink or purple petals, 80–150 stamens with exine, rugulate pollen about $1.4 \mu\text{m}$ thick, a long style similar or exceeding the stamens in height with polyspermous placentas (Demoly and Montserrat, 1993).

Within section *Erythrocistus*, resin excreting glandular trichomes appear in *C. albidus*, *C. creticus* subsp. *creticus*, the short, curled-leaved *C. crispus* (Gulz et al., 1996) and *C. parviflorus*. Peculiarly, *C. creticus* subsp. *eriocephalus* leaves seem to contain only non-glandular trichomes (Paolini et al., 2009). This can explain the different chemical profile between the two subspecies, *C. creticus* subsp. *creticus* (CC) and *C. creticus* subsp. *eriocephalus* (CE). This observation can be of valuable help in applying modern genomic approaches that allow the distinction between the two sub-species in order to isolate and characterize biosynthetic genes leading to the production of active labdane diterpenes present in the former sub-species.

C. albidus has bright purple flowers (June to August), *C. creticus* purplish-pink (mid-April to mid-June) and so are those of *C. crispus* (June to August), while *C. parviflorus* has small light pink flowers (Sweet, 1830).

C. albidus displays ecotypic differentiation, at least when growing in semi-arid climates, being able to adapt the growth of its branches and leaf dimensions, acquiring the greatest growth under plentiful water availability, while slowing growth and tending to phenotypically converge under drier environments (Grant et al., 2005).

The leaves of the Cretan rock rose *C. creticus* exhibit the phenomenon of seasonal dimorphism, as an adjustment mechanism for acclimatization to the Mediterranean climate. During summertime, when water is limited, brachyblasts are developed that have leaves five-times shorter than the ones in winter, with stomata located abaxially inside crypts (Aronne and Micco, 2001). During wintertime, the newly developed dolichoblasts are fourteen times longer, bearing a bigger number of leaves with stomata distributed across the lower surface.

The curled-leaved *C. crispus* reaches only up to 70 cm in height (Sweet, 1830) and is covered by whitish hair (Sweet, 1830). *C. parviflorus* on the other hand, is one of the tall *Cistus* plants, that can reach a height of 1.5 m. According to Guzmán et al. (2009), *C. parviflorus* is actually classified within the white flowered lineage of the *C. salvifolius* species group.

Subgenus II: *Leucocistus* WILLK

Plants in this subgenus carry white flowers with 3–5 sepals, and have exine pollen around 4.2 microns thick, crosslinked, or shallow mesh foveolae and polyspermous placentas (Demoly and Montserrat, 1993).

In section *Ledonia* belong the two most widespread *Cistus* species, *C. monspeliensis* and *C. salviifolius*, together with *C. populifolius*. Characteristics of this section are the five sepals, which are either subequal or the two external are longer, and the style being slightly shorter than the stamens (Demoly and Montserrat, 1993). Both *C. monspeliensis* and *C. salviifolius* carry glandular and non-glandular trichomes, while distinctive to *C. populifolius* is the existence of only the glandular type (Gulz et al., 1996). *C. populifolius* is further divided in subspecies *populifolius* and *major* Dunal.

C. monspeliensis, also known as the Montpellier rock-rose, is characterized by its aromatic leaves and its small white flowers (Angelopoulou et al., 2001a; Kalpoutzakis et al., 2003). It also exhibits seasonal leaf dimorphism with alternating wide and thin late autumn/early winter and thicker late spring/early summer leaves with larger trichome density, while both types coexist on the same plant during early spring (de Dato et al., 2013). Summer leaves have high leaf mass area and tissue density, low leaf surface area and thick adaxial cuticle, traits that contribute to the plants endurance to drought conditions and resistance to fire (Catoni et al., 2012). As expected, long-term experimental drought conditions during the transition to summer leaves can have significant effect on leaf functioning (de Dato et al., 2013). In a relevant study, over-imposed drought resulted in early leaf litter and a reduction of spring-leaf lifespan, thus a shorter vegetative season, which can have a negative effect on *C. monspeliensis*' survival in Mediterranean shrubland (De Dato et al., 2008; de Dato et al., 2013).

Characteristics of section *Ladanium* include three sepals, large petals and an inconspicuous style (Demoly and Montserrat, 1993). It consists of the laurel-leaved *C. laurifolius*, which is the tallest (1–2 even 3 m) and the very short (50–400 cm) flat-leaved Gum *C. ladanifer*, which is further divided in three subspecies, *ladanifer*, *africanus* Dans, and *sulcatus* Demoly. Both species carry non-glandular and resin producing glandular trichomes on the abaxial surface of the leaves (Gulz et al., 1996). In *C. ladanifer*, wavy lamina-forming crypts are arranged on the abaxial side, near which non-glandular trichomes are mostly gathered (Tattini et al., 2007).

Subgenus III: *Hamilioides* (WILLK)

The general characteristics of this subgenus are the three sepals, surrounding small, white petals, 30–40 stamens, grooved or fluted-reticulate exine pollen about 2.8 μm thick, and a short style, slightly exceeded the stamens (Demoly and Montserrat, 1993).

C. clusii, also called Clusius's rock rose, is a vigorously growing shrub, highly resistant to drought (Pugnaire and Lozano, 1997). It belongs to the *C. clusii* group, comprising one of the two white flowered *Cistus* lineage species, together with *C. munbyi*, and is divided in two subspecies, namely subsp. *clusii* and subsp. *multiflorus* Willk (Guzmán et al., 2009).

GEOGRAPHICAL DISTRIBUTION

GENUS *CISTUS* L

Cistus plants are extensively distributed in the Mediterranean region, covering most areas from the Canary Islands and Madeira to Caucasus and Israel, colonizing the Iberian, Apennine, Balcan, Crimean, and Anatolian peninsulas and North Africa. In this review we have studied the ten most prominent *Cistus* species, which are widely spread within the Mediterranean basin (Table S1). The geographical distribution of the species and subspecies can be further explored by visiting the following webpage: Interactive Map of *Cistus* distribution. In the next paragraphs we describe the distribution, diversification and habitat preference of these species.

Subgenus I: *Cistus* L

C. albidus grows in evergreen shrublands, is partially drought-deciduous (Grant et al., 2005) and a non-strict calcicole (Soriano and Gómez Miguel, 2009) that prefers calcareous and basic soils (De Vega et al., 2008) and woodlands with plenty *Pinus* and *Quercus* compost, in dry areas with altitude up to 1200 m (Guzmán and Vargas, 2010). Phylogenetic studies have shown that it is closely related to the endemic species forming the Canarian *Cistus* lineage, while still forming a monophyletic group with two other purple-flowered species exclusive in the Mediterranean basin, *C. creticus* and *C. heterophyllus* (Guzmán and Vargas, 2010).

Several populations of *C. creticus* are spread in central-eastern Mediterranean, including Corsica and Sardinia (Falchi et al., 2009) and the island of Crete in Greece (Demetzos et al., 2002a). Among the three *C. creticus* subspecies identified, subsp. *corsicus* is limited to the islands of Corsica and Sardinia (Falchi et al., 2009). More than twenty five populations of subsp. *creticus* are endemic to the coastal areas of Crete (Greece) (Demetzos et al., 2002a). Subspecies *eriocephalus* is exclusive to the Mediterranean area (Demetzos et al., 2001), mainly located on the islands of Corsica, Sardinia (Paolini et al., 2009), and Crete (Demetzos et al., 1997).

C. crispus is endemic to southern France, Spain, Iberian, and Apennine Peninsulas and to northwest Africa (Guzmán et al., 2009) and grows in clay or stony soils.

The purple-flowered *C. parviflorus* is a distinctive member of the white-flowered species lineage of *Cistus* that seems to have diverged in the Middle Pliocene (3.13 ± 0.08 Ma) (Guzmán and Vargas, 2010). Though it appears that early divergence of *Cistus* species occurred in the western Mediterranean, *C. parviflorus* is distributed exclusively in the eastern Mediterranean (Guzmán et al., 2009; Guzmán and Vargas, 2010). It prefers dry climates and shrublands with calcicolous soils (Guzmán et al., 2009).

Subgenus II: *Leucocistus* WILLK

Species of *Leucocistus* are widespread in the Mediterranean basin and Madeira, the Canary and Balearic Islands, reflecting their successful adaptation and colonization in Mediterranean habitats (Robles and Garzino, 2000; Guzmán and Vargas, 2005).

C. monspeliensis is spread from the western Mediterranean to the Canary Islands and Madeira where it seems to have occurred naturally without any human intervention

(Guzmán and Vargas, 2010). The origin of this colonization and further diversification in the Canaries appears to be on the islands of Tenerife and Gran Canaria that have favorable ecological conditions for the growth of the species, and where *C. monspeliensis* is widely distributed today (Fernández-Mazuecos and Vargas, 2011). Comparative phylogeography was used to demonstrate that the migration of the species to La Gomera and El Hierro occurred via long-distance dispersal from Tenerife to the southwest. *C. monspeliensis* is dominant in evergreen garrigue vegetation, inhabiting acidic, limestone, silicolous and calcareous hills and colonizing areas that are rich in *Quercus* and *Pinus* trees compost or have been disturbed by fire (Angelopoulou et al., 2001a; Guzmán and Vargas, 2005; Guzmán et al., 2009; Catoni et al., 2012). It has also been demonstrated to be a non-strict calcifuge (Soriano and Gómez Miguel, 2009).

C. populifolius inhabits areas of the western Mediterranean basin and prefers volcanic and silicolous soils (Guzmán et al., 2009).

C. salviifolius is the most widely spread species of the genus *Cistus* around the Mediterranean basin. At least three intercontinental colonizations are responsible for its wide distribution, leading to little geographical isolation with high genetic diversity within populations, but no genetic differentiation between the different populations of *C. salviifolius* (Farley and McNeilly, 2000; Fernández-Mazuecos and Vargas, 2010). The factors that caused the dispersion of *C. salviifolius* around the Mediterranean were mostly ecological, such as the climate and the soil. It grows in silicolous and calcicolous soils and occurs on sandy soils of a wide range of habitats (Guzmán et al., 2009), while it is often located within the understorey in wooded areas (Farley and McNeilly, 2000).

Section 2: *Ladanium* (SPACH). Natural habitats of *C. ladanifer* are located exclusively in the western Mediterranean. The subspecies of *C. ladanifer* are distributed in close and overlapping geographical regions. It grows in volcanic and silicolous soils in habitats with dry and hot climate (Guzmán et al., 2009).

The adaptation of this species in dry, hot areas is due to its hairy, amphistomatous, and wavy leaves with stomata mostly concentrated in the crypts formed on the abaxial surface of the leaf (Tattini et al., 2007).

C. laurifolius prefers silicolous soils and mesic and high altitudes with Mediterranean mountain climate. This ecological preference for habitat has isolated the European and African populations, which were produced by a single, eastward migration event (Fernández-Mazuecos and Vargas, 2010).

Subgenus III: *Hamilioides* WILLK

The species in this subgenus are exclusive in the western Mediterranean (Guzmán and Vargas, 2005).

C. clusii is highly efficient in surviving in harsh environments colonizing post-fire and perturbed areas (Pugnaire and Lozano, 1997). It prefers calcicolous soils and dry to semi-arid environments, and can grow in high altitudes, up to 1500 m from the coastline (Guzmán et al., 2009).

CHEMICAL ANALYSES

A large variety of secondary metabolites occurs in different tissues of the 10 *Cistus* species covered in this review. In total, 733 chemical substances have been reported, 397 of which are terpenes (101 monoterpenes, 178 sesquiterpenes, and 118 diterpenes), 162 are of phenylpropanoid nature (128 flavonoids, 17 phenolics, and 12 tannins), 24 hydrocarbons, 35 fatty acids, 36 carbonylic compounds, and 18 phytohormones and vitamins (Tables S3, S4). Specifically, *C. albidus* is one of the most studied species and contains 140 terpenes (34 monoterpenes, 101 sesquiterpenes, and 5 diterpenes), 24 phenylpropanoids (18 flavonoids, 2 phenolics, and 4 tannins), 9 hydrocarbons, 24 fatty acids, 7 carbonylic compounds, and 18 phytohormones and vitamins.

In *C. creticus* subsp. *creticus* 92 terpenes (36 monoterpenes, 35 sesquiterpenes, and 21 diterpenes) and 12 phenylpropanoids-flavonoids have been reported. In *C. creticus* subsp. *eriocephalus* 47 terpenes (consisting of 17 monoterpenes, 19 sesquiterpenes, and 11 labdane-type diterpenes) and 2 carbonylic compounds have been detected. The main secondary metabolites identified in *C. clussi* and *C. crispus* are phenylpropanoids: 23 phenylpropanoids (15 flavonoids, 3 phenolics, and 5 tannins) and one labdane-type diterpene for the former and 10 phenylpropanoids (6 flavonoids, 2 phenolics, and 2 tannins) for the latter. In *C. ladanifer*, 72 terpenes (47 monoterpenes, 18 sesquiterpenes, and 7 labdane-type diterpenes), 43 phenylpropanoids (27 flavonoids, 3 phenolics, and 12 tannins) plus an additional 6 carbonylic compounds have been identified.

C. laurifolius is a main source of phenylpropanoids (44 flavonoids, 6 phenolics, and 7 tannins), and also contains 4 terpenes (1 labdane-type diterpene and 3 clerodanes) and 1 carbonylic compound. A plethora of studies conducted using *C. monspeliensis* revealed a high content of secondary metabolites, especially terpenoids (22 monoterpenes, 33 sesquiterpenes, and 52 diterpenes). It also produces 17 phenylpropanoids (6 flavonoids, 6 phenolics, and 4 tannins), 20 hydrocarbons, 17 fatty acids, and 10 carbonylic compounds.

In *C. parviflorus*, 99 terpenes (17 monoterpenes, 44 sesquiterpenes, and 38 diterpenes), 19 phenylpropanoids (17 flavonoids and 2 phenolics), 8 hydrocarbons, 5 fatty acids, and 7 carbonylic compounds were identified. *C. populifolius* contains 10 clerodane diterpenes, 10 phenylpropanoids (out of which 1 flavonoid, 1 phenolic compound, and 8 tannins). Another species rich in secondary metabolites, terpenes and phenylpropanoids, is *C. salviifolius*: terpenes consist of 32 monoterpenes, 85 sesquiterpenes and 43 diterpenes, while the phenylpropanoid content includes 39 flavonoids, 8 phenolic compounds, and 9 tannins. Finally, 14 hydrocarbons, 5 fatty acids, and 22 carbonylic compounds complete the metabolic profile of the species. The following sections are devoted to the main chemical constituents of *Cistus* species.

TERPENES

Metabolite content and volatiles in *Cistus* are influenced by several factors including diurnal, seasonal, ecological, drought, temperature, plant age, and precipitation. Also, depending on the type of trichomes they contain, *Cistus* species can be high producers of

monoterprenes and sesquiterpenes, while others in diterpenes and clerodanes.

Monoterpenes

Annual presence of several monoterpenes, including α -pinene and limonene, was observed in *C. albidus* grown in Catalonia, Spain (Llusà et al., 2010). Minor amounts were identified mostly in flower tops and other tissues (leaves, petals, sepals) collected from plants that grow in nature, in Italy (Maccioni et al., 2007). High amounts of thymol and carvacrol were exclusive to the pollen (Maccioni et al., 2007). Only a small proportion of oxygenated monoterpenes (0.2%), but no monoterpene hydrocarbons, was identified in *C. albidus* leaves that grow in nature in France (Paolini et al., 2008). In another work, many monoterpenes, including 3-carene, camphene, *cis*-linalool oxide, *cis*-thujone, tricyclene, verbenone, and α -thujene and traces of borneol were detected in Mediterranean *C. albidus* leaves (Ormeño et al., 2007). Similarly, many monoterpenes and oxygenated monoterpenes were found in *C. creticus* subsp. *creticus* leaf and essential oil, collected from different regions of Crete in Greece (Demetzos et al., 1994b, 1995). The most abundant leaf volatiles collected from aerial parts of Cretan *C. creticus* subsp. *eriocephalus* from different regions of Corsica (France) and North Sardinia were monoterpenes, especially myrcene and limonene (Paolini et al., 2009). Several monoterpenes were also present in essential oils, but in significantly smaller concentrations (Demetzos et al., 1997). Similarly, monoterpene compounds constitute the majority of terpenes identified in essential oils of several *C. ladanifer* populations found in northern Portugal (Ramalho et al., 1999; Gomes et al., 2005; Teixeira et al., 2007), Spain (Alías et al., 2012), Morocco (Zidane et al., 2013), and south France (Mariotti et al., 1997; Robles and Bousquet-Mélou, 2003).

The monoterpene phenol carvacrol was a major constituent of Tunisian *C. monspeliensis* leaves and essential oils, while other polyphenolic compounds such as diisobutyl ester (phthalic acid) and benzyl benzoate were also strongly represented (Jemia et al., 2013; Loizzo et al., 2013). Moreover, carvacrol and α -terpineol were the only monoterpene compounds isolated from leaf essential oil of *C. monspeliensis* plants found in Crete (Greece) (Angelopoulou et al., 2001a, 2002), while several phenolic acid derivatives were identified in plants grown in Spain (Barrajón-Catalán et al., 2011). Several monoterpenes, including hydrocarbons and oxygenated monoterpenes were identified as the major components in leaves and essential oils of Tunisian and French *C. monspeliensis* plants (Rivoal et al., 2010; Jemia et al., 2013; Loizzo et al., 2013). Monoterpenes were 14.47% of the total essential oil extracted from Cretan *C. parviflorus* (Demetzos et al., 1990b). The highest concentration of monoterpenes in the essential oil from *C. parviflorus* plants grown in Crete were mainly oxygenated monoterpenes while no traces of monoterpene hydrocarbons were reported. Carvacrol was identified as the major constituent in all samples (Angelopoulou et al., 2001b). Mostly oxygenated monoterpenes and only a small fraction of monoterpene hydrocarbons have been detected in a large number of *C. salviifolius* populations from Crete (Greece) and Tunisia (Demetzos et al., 2002b; Loizzo et al., 2013).

Sesquiterpenes

Chemical analysis of *C. albidus* tissue and essential oil preparations in north-eastern Spain, France and Italy showed high content of sesquiterpenes, in particular, β -sesquiphellandrene, β -caryophyllene, β -bourbonene, α -zingiberene, and germacrene D (Robles and Garzino, 1998; Maccioni et al., 2007; Paolini et al., 2008; Llusà et al., 2010). Among them, α -zingiberene and germacrene D were the most abundant, while *epi*-10- γ -eudesmol was detected uniquely in flowers, 1-*epi*-cubenol only in leaves, and β -himachalene exclusively in flower tops (Maccioni et al., 2007). In a study conducted on Mediterranean *C. albidus* (Ormeño et al., 2007), germacrene D, *ar*-curcumene and *allo*-aromadendrene constituted the highest content of emissions from leaves. Analyses of essential oils secreted from the resin of the Cretan plant *C. creticus* subsp. *creticus* revealed the presence of several sesquiterpenes and oxygenated sesquiterpenes (Demetzos et al., 1994b, 1999). The sesquiterpene fraction was the largest in the essential oils of *C. creticus* subsp. *eriocephalus* endemic in the Cretan flora in Greece (Demetzos et al., 1997).

High concentrations of sesquiterpenes have been measured in aerial parts and essential oils of several *C. ladanifer* populations in France, Portugal, Spain and Morocco, with vidiflorol being the most abundant molecule (Mariotti et al., 1997; Ramalho et al., 1999; Robles and Bousquet-Mélou, 2003; Gomes et al., 2005; Teixeira et al., 2007; Zidane et al., 2013). Analysis of essential oil composition of *C. monspeliensis* grown in Tunisia led to the identification of accountable amounts of oxygenated sesquiterpenes and sesquiterpene hydrocarbons (Loizzo et al., 2013). Another study conducted with plants at the same site detected a large variety of sesquiterpenes but at low concentrations (Jemia et al., 2013). Several sesquiterpenes were also isolated from *C. monspeliensis* plants naturally growing in areas of France and Greece (Robles and Garzino, 2000; Angelopoulou et al., 2001a, 2002; Rivoal et al., 2010). Sesquiterpene compounds identified in *C. monspeliensis* plants located in Crete (Greece), accounted for a total content of 38.5% in leaf and 6.6% in fruit essential oil (Angelopoulou et al., 2001a).

Mainly oxygenated sesquiterpenes were identified in high percentages in the essential oil of nine populations of *C. parviflorus* endemic to the island of Crete, with caryophyllene oxide and α -*epi*-cadinol being the most abundant (Angelopoulou et al., 2001b). High concentrations of sesquiterpenes were also found in aerial parts and essential oils of several *C. salviifolius* Cretan and Tunisian populations, among which oxygenated sesquiterpenes were the most abundant (Demetzos et al., 2002b; Loizzo et al., 2013).

Diterpenes

Only a small fraction of the essential oil composition of *C. albidus* plants collected in south France and Spain contained diterpenes (Paolini et al., 2008; Llusà et al., 2010). Among them the largest concentration was measured for the labdane-type compound 13-*epi*-manoyl oxide (Paolini et al., 2008). Chemical analysis of the aerial parts and essential oil of *Cistus creticus* subsp. *creticus* plants native to Crete, Greece, revealed the presence of several labdane-type diterpenes (especially manoyl oxide and 13-*epi*-manoyl oxide) (Demetzos et al., 1990a, 1994b,c, 1995,

1999, 2002c; Anastasaki et al., 1999; Falara et al., 2010). The first metabolomic analysis of isolated trichomes from this species (Falara et al., 2010) detected the presence of several labdane-type diterpenes (8,13-*epoxy*-15,16-dinorlabd-12-ene, 13-*epi*-manoyl oxide, 3 β -hydroxy-13-*epi*-manoyl oxide, labd-7,13-dien-15-ol, labd-7,13-dien-15-yl acetate, 3 β -acetyl-13-*epi*-manoyl oxide, labd-13-en-8 α ,15-diol, and labd-13-en-8 α -ol-15-yl acetate). Further experiments in this study demonstrated that the production of labdane-type diterpenes in *Cistus* is trichome-specific (Falara et al., 2010).

The labdane-type diterpenes manoyl oxide (9.9%) and 13-*epi*-manoyl oxide (3.4%) constituted a significant fraction of components in Cretan *C. creticus* subsp. *eriocephalus* leaves' extracts (Demetzos et al., 1997). The diterpenes that comprise the major diterpene fraction of Spanish *C. ladanifer* are 6-acetoxy-7-oxo-8-labden-15-oic acid, 7-oxo-8-labden-15-oic acid and oxocatic acid (Alías et al., 2012), while more labdane-type diterpenes were found in other studies conducted in France, Portugal and Spain (Mariotti et al., 1997; Gomes et al., 2005; Tomás-Menor et al., 2013).

The labdane-type diterpene 6 β ,8-dihydroxy-*ent*-13*E*-labden-15-oic acid (laurifolic acid) (De Pascual Teresa et al., 1986) as well as the clerodanes salmantic acid and its methyl ester, salmantid-ol (de Pascual Teresa et al., 1983) were isolated and characterized from *C. laurifolius* extracts. Leaves and essential oils from *C. monspeliensis* plants collected in France, Greece, Morocco, and Tunisia were rich in diterpenes (Berti et al., 1967; Robles and Garzino, 2000; Angelopoulou et al., 2001a, 2002; Oller-López et al., 2005; Loizzo et al., 2013). The labdane-type diterpenes 13-*epi*-manoyl oxide, manoyl oxide and epimers were the most abundant in all studies. In *C. monspeliensis* leaves from plants grown in Tunisia, diterpenes constituted only a small fraction (3.8%) of the total secondary metabolite content (Jemia et al., 2013). The analysis of the aerial parts and essential oils of *C. monspeliensis* plants grown in Greece revealed the existence of several clerodanes (Berti et al., 1970; Angelopoulou et al., 2001a; Demetzos et al., 2001; Kalpoutzakis et al., 2003). The labdane-type diterpenes manoyl oxide mixture of isomers and 13-*epi*-manoyl oxide were detected in all the nine populations of *C. parviflorus* grown in Crete (Angelopoulou et al., 2001b). In a study by Demetzos et al. (1990b), 37.78% of the essential oil constituents were diterpene compounds. Clerodanes were the only diterpenes detected and characterized from *C. populifolius* (Urones et al., 1994, 1995).

Diterpenes were also a significant fraction of the metabolites identified in *C. salviifolius* populations. The most abundant diterpene in plants endemic in Tunisia was manoyl oxide (Loizzo et al., 2013). In Cretan populations, manoyl oxide and 13-*epi*-manoyl oxide were also detected, but *cis*-ferruginol was the most abundant metabolite (Demetzos et al., 2002b).

PHENYLPROPANOIDS

Flower scent is a vital strategy that plants use for attracting pollinators and ensuring their reproduction and survival (Gang, 2005). Volatile phenylpropanoids have a significant role among the compounds emitted by the plants in order to contribute to this aroma. Moreover, as a defensive mechanism of the plants against high solar radiation and drought, the content of the

antioxidant flavonoids in *Cistus* species is highly variable. In most species, increased concentrations of flavonoids are observed during summer and in younger leaves. In addition to emissions, light, especially UV-B radiation, positively influences in a periodic manner the absorbing capacity of epicuticular phenolic substances in *C. creticus*, contributing to the plants photoprotective mechanism (Stephanou and Manetas, 1997). High concentrations of the non-volatile group of tannins have also been measured in various *Cistus* species, including the hydrolysable gallic and ellagic acids and ellagitannins (Barrajón-Catalán et al., 2011).

Flavonoids

Several flavonoids, including quercetin, myricetin, kaempferol, apigenin and their derivatives, were isolated from leaves and resin of Cretan *C. creticus* subsp. *creticus* (Demetzos et al., 1989, 1990a). The same compounds were also detected in young leaves of Mediterranean *C. laurifolius* (Orhan et al., 2013), aerial parts of Spanish *C. salviifolius* plants (Danne et al., 1994; Saracini et al., 2005; Qa'Dan et al., 2006; Barrajón-Catalán et al., 2011; Loizzo et al., 2013; Tomás-Menor et al., 2013) and aqueous extracts of Spanish *C. crispus* plants (Barrajón-Catalán et al., 2011). The anti-oxidant polyphenolic flavonoids catechin, gallocatechin and several of their derivatives, as well as other flavonols, flavanols, flavonoid glycosides and proanthocyanidin compounds have been identified in *C. albidus* leaves (Qa'Dan et al., 2003; Barrajón-Catalán et al., 2011; Tomás-Menor et al., 2013). Flavonoid related compounds were detected in aerial parts of *C. clusii* grown naturally in Spain, including kaempferol diglucoside and the (–)-(epi)gallocatechin-(epi)gallocatechin dimer (Barrajón-Catalán et al., 2011). The latter compound was also identified in the leaves of field-grown *C. clusii* plants together with significant amounts of flavan-3-ols and the simpler proanthocyanidins (Hernández et al., 2011).

Kaempferol and its derivatives were also detected in *C. ladanifer*, together with several flavonoids belonging to the groups of flavones, flavonols and flavon-3-ols (Chaves et al., 1993; Fernández-Arroyo et al., 2010; Barrajón-Catalán et al., 2011). In addition, plants of this species accumulate these flavonoids (particularly acylated kaempferol 3-O-glycosides) in the non-glandular trichomes.

Many biologically active flavonoids were also identified in tissue extracts of *C. laurifolius* (Vogt et al., 1988; Vogt and Gerhard Gul, 1994; Enomoto et al., 2004; Küpeli et al., 2006; Sadhu et al., 2006; Küpeli and Yesilada, 2007; Akkol et al., 2012; Orhan et al., 2013). The flavonol aglycones quercetin 5,3'-dimethyl ether and quercetin 3,5,3'-trimethyl ether, detected in the leaf resin of young *C. laurifolius* leaves, have been identified and characterized (Vogt et al., 1988). Variable amounts and types of flavonoid compounds were identified in hexane leaf extracts of *C. monspeliensis* plants naturally growing in Spain and Tunisia (Pomponio et al., 2003; Barrajón-Catalán et al., 2011; Jemia et al., 2013), while only few flavonoid substances were isolated from *C. populifolius* aqueous extracts (Barrajón-Catalán et al., 2011). *C. parviflorus* leaves' resin contained several flavonoids including kaempferol, quercetin methyl ethers and 6- and 8-O-methylated flavonols (Vogt et al., 1987).

Phenolic acids and tannins

Gallic acid and hexahydroxydiphenyl-glucose, together with variable gallic acid-derived hydrolysable ellagitannins were identified in *C. albidus*, *C. clusii*, *C. crispus*, *C. creticus*, *C. ladanifer*, *C. laurifolius*, *C. monspeliensis*, *C. populifolius*, and *C. salvifolius* leaf samples collected in Spain, with evident ecological variation (Barrajón-Catalán et al., 2011). *C. laurifolius*, is a high producer of tannins throughout the year, especially in younger leaves (Ammar et al., 2004). Several phenolic acids and derivatives have also been detected in aerial parts of *C. albidus*, *C. clusii*, *C. crispus*, and *C. ladanifer* (Ramalho et al., 1999; Qa'Dan et al., 2003; Teixeira et al., 2007; Barrajón-Catalán et al., 2011). In addition, high amounts of elemicin and several phenolic compounds were detected in *C. salvifolius* leaves (Loizzo et al., 2013).

HYDROCARBONS

Among volatile organics isolated from leaves of *C. albidus* collected from southern Catalonia, Spain were docosane, octacosane, and tetracosane (Llusà et al., 2010). Linear hydrocarbons including *n*-tetradecane and *n*-hexadecane, were identified exclusively in the pollen and not in other flower parts or leaves of *C. albidus* growing in Italy in late spring (Maccioni et al., 2007). Only tricosane was detected in the essential oil of *C. albidus* plants growing in France (Paolini et al., 2008). Several hydrocarbons such as heptacosane, nonacosane, pentacosane, and tricosane were also produced by *C. monspeliensis* endemic in the South of France, Greece, and Tunisia (Robles and Garzino, 2000; Angelopoulou et al., 2001a; Jemia et al., 2013; Loizzo et al., 2013). Tetracosane, pentacosane, pentadecane, neophytadiene, heptadecene, docosane, heneicosane, and dodecane were all detected in *C. parviflorus* (Angelopoulou et al., 2001b), while neophytadiene and pentacosane were identified in several Greek and Tunisian populations of *C. salvifolius* (Demetzos et al., 2002b; Loizzo et al., 2013).

FATTY ACIDS

Several fatty acids were identified in aerial parts and essential oils of *C. albidus* growing in north-eastern Spain, including tetracosanoic acid and pentacosanoic acid (Llusà et al., 2010; Müller et al., 2014). Fatty acid composition of *C. albidus* seeds was studied in both young and old plants. High concentrations of linoleic acid, and generally polyunsaturated as well as very long chain saturated fatty acids were found in seeds of the older plants (Müller et al., 2014). Various fatty acids and esters were identified as minor or major components of aerial parts and essential oil content of French, Greek, and Tunisian *C. monspeliensis*, *C. creticus* subsp. *creticus*, and *C. salvifolius* plants (Demetzos et al., 1994b, 2002b; Robles and Garzino, 2000; Angelopoulou et al., 2001a; Jemia et al., 2013; Loizzo et al., 2013).

CARBONYLIC COMPOUNDS

Aliphatic aldehydes including octanal, nonanal, decanal and 6-methyl-5-hepten-2-one were present only in the pollen and not in other flower parts or leaves of *C. albidus* plants growing in Italy in late spring (Maccioni et al., 2007). Nonanal, decanal, undecanal, and dodecanal were detected in the essential oil of *C. albidus* plants growing wild in France (Paolini et al., 2008). Nonanal and

β -ionone were also detected in *C. creticus* subsp. *eriocephalus* leaves. Among the secondary metabolites produced by French, Greek and Tunisian *C. monspeliensis* plants, the norisoprenoids (E)- β -damascenone and members of the ionone family, used in the fragrance industry, as well as several other carbonylic compounds were identified (Robles and Garzino, 2000; Angelopoulou et al., 2001a, 2002; Demetzos et al., 2002b; Jemia et al., 2013; Loizzo et al., 2013).

PHYTOHORMONES AND VITAMINS

The production of phytohormones and vitamins were studied in *C. albidus* seeds (Müller et al., 2014), leaves, and flowers (Oñate and Munné-Bosch, 2010), in relation to plant maturity. The main vitamin E compound in *C. albidus* seeds was α -tocopherol, whose content was higher in mature plants than the younger ones (Müller et al., 2014). Similarly, seeds of mature plants had higher concentrations of both salicylic acid and jasmonic acid, which was not the case in flowers and leaves, where no concentration differences were observed between the age groups (Oñate and Munné-Bosch, 2010). The adaptability of the species to drought involved an increase in abscisic (ABA) and ascorbic acid (AA) levels, as well as leaf H_2O_2 concentrations, localized mainly in mesophyll cell walls, xylem vessels, and differentiating sclerenchyma cells (Jubany-Marí et al., 2009). Recovery from drought implicated the readjustment of ABA, AA, and H_2O_2 to their basal concentrations (Jubany-Marí et al., 2009). Moreover, efficient acclimation to drought was only achievable in the first of three consecutive cycles of experimental drought and re-watering applications of *C. albidus* plants (Galle et al., 2011).

C. creticus is also a highly drought-resistant plant, and was used as a model to study drought-induced changes in the expression of genes encoding enzymes involved in isoprenoid biosynthesis, as well as the corresponding metabolites (chlorophylls, carotenoids, tocopherols, and abscisic acid) and endogenous concentrations of other growth regulators (jasmonic and salicylic acids, JA and SA, respectively) (Munné-Bosch et al., 2009). Among the genes studied, those encoding homogentisate phytyl-transferase (HPT) and 9-*cis*-epoxycarotenoid dioxygenase (NCED) were induced even at early stages of drought, and were strongly correlated to the levels of the corresponding metabolites. The simultaneous increase in concentrations of ABA and α -tocopherol (but not JA and SA), led the authors to suggest that the genes encoding HPT and NCED may play a key role in drought stress resistance by modulating ABA and tocopherol biosynthesis (Munné-Bosch et al., 2009).

BIOLOGICAL FUNCTIONS

Various preparations from *Cistus* species have traditionally been used as remedies in folk medicine around the Mediterranean basin, especially in Greece, Italy, Spain, and Turkey. The targeted conditions and diseases include anxiety, arthrosis, asthma, bronchitis, various types of cancer, bacterial and fungal infections, cardiopathies, catarrh, corn, diarrhea, duodenitis, dysentery, dyspnea, fracture, gastrositis, headache, hepatosis, hernia, hysteria, induration, infection, inflammation, insomnia, leukorrhea, myalgia, neuralgia, osteoarthritis, polyp, proctosis, rhinosis, sore, spasm, splenosis, ulcer, uterositis (Duke et al., 2008). A considerable amount of studies have thus explored the pharmacological

properties of the resin secreted by *Cistus* leaves. These properties include allergenic, anti-aggregant, anti-leukemic, anti-oxidant, anti-peroxidant, anti-proliferant, anti-radicular, antiseptic, anti-ulcer, astringent, bactericide, candidicide, cardio-protective, cytotoxic, dermo-protective, dipeptidylpeptidase-IV inhibitor, alanyl-aminopeptidase inhibitor, diuretic, emmenagogue, expectorant, fungicide, gastro-protective, hemostat, myorelaxant, nervine, purgative, revulsive, sedative, spasmolytic, stimulant, vulcenary (Duke et al., 2008). Below, we discuss some of the most studied biological functions of *Cistus* species and the chemical nature of the potent corresponding compound groups produced by the plants.

ANTIBACTERIAL, ANTIFUNGAL

Organic and aqueous leaf extracts of *C. monspeliensis*, and also *C. villosus* (= *incanus*), growing naturally in Morocco and Tunisia were shown to have antimicrobial and antifungal properties that were mostly active against *Staphylococcus aureus*, *Enterococcus hirae*, and *Pseudomonas aeruginosa* and the yeast *Candida glabrata* (Bouamama et al., 2006). Flower extracts of *C. monspeliensis* were active against gram-positive bacteria species of genus *Staphylococcus* and had significant growth-inhibitory effects on *Staphylococcus epidermis* (Sassi et al., 2007). Furthermore, a *cis*-clerodane diterpene isolated in large amounts and characterized from *C. monspeliensis* was very active against *Staphylococci* species and had four times higher activity than the labdane-type diterpene sclareol (Kolocouris et al., 2001).

Labdane-type diterpenes mainly represented by *ent*-13-*epi*-manoyl oxide, manoyl oxide and its isomers were found in significant concentrations in hexane extracts of leaves (16.6%) and fruits (25%) of *C. monspeliensis* growing in Crete, Greece (Angelopoulou et al., 2001a). Antimicrobial and antifungal activities of *C. creticus* subsp. *creticus* and subsp. *eriocephalus* have been evaluated (Chinou et al., 1994; Demetzos et al., 1995, 1997; Anastasaki et al., 1999; Bouamama et al., 1999; Hutschenreuther et al., 2010). The essential oil extracted from leaves of *C. creticus* subsp. *eriocephalus*, which has manoyl oxide and 13-*epi*-manoyl oxide as its main constituents, exhibited a rather weak activity against *E. coli* and *P. aeruginosa*, moderate against *Candida albicans*, *Micrococcus luteus*, and *S. epidermidis*, and most active against *S. aureus* and *Bacillus subtilis* (Demetzos et al., 1995), while the volatile oil fraction had a high *in vitro* activity against *Borrelia burgdorferi sensu stricto* (Hutschenreuther et al., 2010). The antimicrobial activity of manoyl oxide extracts from *C. creticus* subsp. *creticus* fruits, leaves and resin was found dose-dependent against gram-positive *Staphylococci* species, inactive against gram-negative bacteria (Anastasaki et al., 1999; Demetzos et al., 1999, 2001, 2002c).

Among several tested labdane-type diterpenes, labd-13-*en*-8 α ,15-diol was the only diterpene reported to be active against *Candida albicans*, while labd-7,13-dien-15-ol did not exhibit any antibacterial or/and antifungal activity (Chinou et al., 1994; Bouamama et al., 2006). Several semisynthetic labdane-type diterpenes from the resin "ladano" of *C. creticus* were reported for their antimicrobial activity both against gram positive and gram negative bacteria and also against pathogenic fungi with the highest effects exhibited by two chloroethyl carbamidic

esters derivatives (Kalpoutzakis et al., 2001). *C. ladanifer* and *C. populifolius* aqueous extracts had antibacterial activity against *E. coli* (Barrajón-Catalán et al., 2010).

Antimicrobial properties have been demonstrated for several phenolic monoterpenes, such as thymol and carvacrol, and other carbonylic and phenolic compounds identified among *C. creticus* and *C. albidus* volatiles (Maccioni et al., 2007; Hutschenreuther et al., 2010). Phenolic compounds present in extracts from *C. ladanifer* aerial tissues displayed antifungal activity against *Candida* species (Barros et al., 2013) in a dose-dependent manner (Barrajón-Catalán et al., 2010). Strong antimicrobial activity against gram-positive bacteria was also demonstrated for *C. ladanifer*, and against gram-negative bacteria for *C. populifolius* (Barrajón-Catalán et al., 2010).

ANTIVIRAL

The polyphenol rich extract CYSTUS052 derived from *C. incanus* was shown to exhibit potent anti-influenza virus activity without causing toxic side effects or inducing viral resistance (Ehrhardt et al., 2007). The first clinical study showed that *C. incanus* extract (CYSTUS052) could be applied for the treatment of upper and lower respiratory tract infections (Kalus et al., 2009). Moreover, *C. ladanifer* and *C. populifolius* extracts were able to inhibit the replication of the vesicular stomatitis virus (VSV) (Abad et al., 1997).

ANTIOXIDANT

Cistus species rich in phenolic compounds, especially flavonoids, demonstrate significant antioxidant properties. Preparations of *C. creticus*, *C. incanus*, *C. libanotis*, *C. salvifolius*, *C. monspeliensis*, *C. parviflorus*, *C. laurifolius*, *C. ladanifer*, and *C. populifolius* aqueous extracts were able to generate strong antioxidant activities in a dose-dependent manner, using several free radical scavenging methods (Attaguile et al., 2000; Sadhu et al., 2006; Teixeira et al., 2007; Amensour et al., 2010; Barrajón-Catalán et al., 2010; Alsabri et al., 2012; Loizzo et al., 2013). Among all studied species, *C. monspeliensis* appeared to have the highest antioxidant activity (Attaguile et al., 2000, 2004; Loizzo et al., 2013). Potential protective and antioxidant activity was suggested for the pollen of *C. incanus*, after evaluating the induction of anti-estrogenic properties and differential expression of genes involved in the apoptosis pathway and chemotaxis of mice fed with rich in pollen bees in Croatia (Sarić et al., 2009).

Plant-derived remedies for human use need to be carefully prepared in order to result in active antioxidant substances. Indeed, *C. incanus* beverages exhibit decreased amounts of phenolic substances and reduced antioxidant activity if an incorrect selection of brewing process parameters (brewing water, temperature, and duration) is made (Riehle et al., 2013).

CYTOTOXIC/ANTICANCER

Cytotoxic activity of shoot extracts from an *in vitro* culture of *C. creticus* subsp. *creticus* against human HeLa cells was shown (Skorić et al., 2012). Shoot extracts (rich in labdane diterpenes) were able to exert cytotoxic activity on HeLa (cervix), MDA-MD-453 (breast), and FemX (melanoma) cancer cells. Cytotoxic and antitumor activities of the bioactive, highly lipophilic, and naturally produced labdane-type diterpenes was improved by

incorporating them in liposomal formulations, a process that constitutes these compounds suitable for testing in *in vivo* experiments (Kyrikou et al., 2005; Matsingou et al., 2005; Hatziantoniou et al., 2006). The labdane-type diterpene sclareol, which is used today as a certified drug, has antitumor activity against human breast cancer cell lines and enhances the activity of known anticancer drugs (Dimas et al., 2006). Sclareol is activated through a p53-independent mechanism that targets the G₁ phase of the cell cycle and therefore apoptosis of human cancer cells is induced through the activation of caspases (Mahaira et al., 2011).

Among nine labdane-type diterpenes isolated from the resin of *C. creticus* subsp. *creticus*, labd-13-en-8 α -ol-15-diol was active against 13 of the 14 cell lines, while labd-7,13-dien-15-ol showed activity only in HL60 human promyelocytic leukemia cells (Dimas et al., 1998). Pure *ent*-13-*epi*-manoyl oxide and mixtures of manoyl oxide isomers isolated from *C. monspeliensis* and *C. creticus* aerial parts of plants from Crete were shown to exert moderate cytotoxic activity (Angelopoulou et al., 2001a; Demetzos et al., 2001). Their activity was evaluated against nine leukemic cell lines, where all tested compounds caused growth inhibition at the highest tested concentration of 10⁻⁴ M (Angelopoulou et al., 2001a). In a previous study, however, *ent*-13-*epi*-manoyl-oxide did not show any antiproliferative effect on the tested human leukemia cell lines (Demetzos et al., 1994a). A dose- and time- dependent inhibition of DNA synthesis by *ent*-3 β -hydroxy-13-*epi*-manoyl oxide, was associated with its conversion to a thiomidazolide derivative (Dimas et al., 1999). Both sclareol and the thiomidazolide derivative induced apoptosis of T-cell leukemic cell lines (Dimas et al., 2001). A number of labdane-type diterpenes isolated from aerial parts of *C. creticus* in Greece, including sclareol, were tested *in vitro* for their cytotoxic activity against human rhinopharynx cancer, murine leukemia and human bronchial epidermoid carcinoma cell lines (Chinou et al., 1994). Among the tested diterpenes, labd-13-en-8 α -ol-15-diol was the only one unable to exhibit nearly any cytotoxic activity. Labd-7,13-dien-15-ol, 8,13-epoxylabd-14-ene (manoyl oxide), 13-*epi*-8,13-epoxylabd-14-ene (13-*epi*-manoyl oxide) caused moderate inhibition against the proliferation of the cell lines, while labd-13-en-8 α -ol-15-yl acetate, labd-14-en-8,13-diol (sclareol) and 13-*epi*-sclareol were highly active (Chinou et al., 1994). Moreover, the *cis*-clerodane (+)-19-acetoxy-*cis*-clerodan-3-en-15-oic acid, isolated from leaf extracts, was tested against several human leukemic lines and did not exhibit any activity (Demetzos et al., 2001). However, none of the diterpenes tested in the above-mentioned studies from Cretan *C. creticus* had any anti-inflammatory activity or any effect on skin repair (Demetzos et al., 2001).

Three flavonoids were also tested against eleven leukemic cell lines. Myricetin had no activity, a myricetin ether that was isolated from the hexane extract of *C. monspeliensis* exhibited significant cytostatic and cytotoxic activities, and its 3',5'-diacetyl derivative, which was chemically synthesized, had lower cytotoxic activity (Dimas et al., 2000).

MYORELAXANT

Cistus extracts have been traditionally used in Mediterranean countries for the treatment of diarrhea, peptic ulcers and as

antispasmodic agents. Myorelaxant effects of *C. incanus* and *C. monspeliensis* aqueous extracts were illustrated on strips of longitudinal smooth muscle of rat ileum and aorta in a concentration-dependent and reversible manner (Attaguile et al., 2004). The authors attributed the inhibition of intestinal mobility by polyphenolic compounds to the high concentrations of flavonoids produced by *Cistus* plants (Attaguile et al., 2004). Moreover, *C. populifolius* and *C. ladanifer* aqueous extracts were evaluated *in vitro* on animal models and their significant, dose-dependent spasmolytic (Sánchez de Rojas et al., 1995) and analgesic effects (de Andrés et al., 1999) were demonstrated.

TOXICITY

Some secondary metabolites produced by *Cistus* sp. exert toxic effects on mammals. The most toxic compounds are gallic acid and tannins, which are detrimental to liver and kidneys. Several cases of lethal toxicoses in cattle have been reported, caused by ingestion of *Cistus* sp., including *C. salviifolius* (Yeruham et al., 2002). Convulsions and lipofuscinosis in the central nervous system have been reported in sheep as directly linked to grazing on *Cistus* sp. (Riet-Correa et al., 2009). In addition, flavonoid-rich extract of *C. laurifolius* was responsible for degenerative effects on the periferic nerval system causing convulsive syndrome in mice (Bregante et al., 1981).

As discussed earlier in this section, many secondary metabolites produced by *Cistus* sp. exhibit toxicity and therefore can be used in low concentrations in order to exert their biological functions. Direct consumption of the plants or their extracts (as tea infusions) in large amounts can become harmful and even lethal.

BIOSYNTHESIS OF COMPOUNDS OF INTEREST

BIOSYNTHETIC PATHWAY FOR TERPENES INCLUDING LABDANE-TYPE DITERPENES

Isoprenoids are one of the largest classes of metabolites with more than 50,000 representatives identified to date in existing organisms with a central role in both primary and specialized metabolism (Thulasiram et al., 2007). Despite the fact that the building block of all isoprenoids is a C₅ isoprene unit, they exhibit an extensive diversity in terms of their size and structure that reflects a wide diversity in their physiological roles. Isoprene is the smallest known 5-carbon terpene, a volatile emitted by the foliage of many tree species. Isoprene emission is considered to enhance tolerance against heat stress and to confer resistance to reactive oxygen species. Monoterpenes are C₁₀-carbon, highly volatile terpenes that contribute to plant odors; they are among the main constituents of flower aromas with an essential role in pollinator attraction. Sesquiterpenes (C₁₅) are also volatile compounds known to play a signaling role in plant defense mechanisms, directly as repellants for herbivores or indirectly through the attraction of herbivore predators and/or pathogens. Diterpenes (C₂₀) are produced by plants for defense purposes (phytoalexins), in addition to serving as precursors of plant hormones (e.g., tocopherols and gibberellins). Triterpenes (C₃₀) are synthesized by the head to tail condensation of C₁₅-carbon terpenes and they are the precursors of phytosterols, brassinosteroids, phytoalexins and waxes. Carotenoids are C₄₀-carbon terpenes that contribute to the pigment of flowers and fruit and play an essential role in

pollination and seed dispersal as well as protection against UV light. Higher orders of isoprenoids, the polyprenyls, are used for co-translational modification of proteins called prenylation that promotes their interaction with cell membranes.

There are two distinct pathways responsible for the biosynthesis of terpenes (Figure S1) in the plant cell (Vranová et al., 2012). The mevalonate pathway (MVA) that operates in the cytosol and starts from acetyl-CoA, and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway that operates in the plastid and is initiated by C-5 sugars. Their names come from intermediate precursors of the pathways; the mevalonate in the cytosolic pathway and the 2-C-methyl-D-erythritol 4-phosphate in the plastidial pathway. The final product of both metabolic routes is isopentenyl pyrophosphate (IPP), the universal precursor of all isoprenoids produced in the plant cell. IPP gets isomerized to dimethylallyl pyrophosphate (DMAPP) by the enzyme isopentenyl pyrophosphate isomerase.

In the MVA pathway, acetyl-CoA from the citric acid cycle undergoes condensation with another acetyl-CoA to produce acetylacetyl-CoA via the enzyme acetyl-CoA transferase (AAT). Next, HMG-CoA synthetase catalyzes the condensation of one more molecule of acetyl-CoA with acetylacetyl-CoA to form 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA). HMG-CoA is then reduced to mevalonate by the HMG-CoA reductase (HMGR) using NADPH as co-factor. Mevalonate kinase (MK) activity yields 5-phospho-mevalonate, which then gets decarboxylated to produce IPP. IPP is then isomerized to DMAPP by the enzyme isopentenyl pyrophosphate isomerase (Flesch and Rohmer, 1988; Rohmer et al., 1993).

The first step of the plastidial pathway involves the condensation of pyruvate with 3-phosphoglycerinaldehyde to produce 1-deoxy-D-xylulose 5-phosphate (DXP) (McGarvey and Croteau, 1995). This reaction is catalyzed by 1-deoxy-5D-xylulose synthase (DXS) that functions as a transacetylase (Lange et al., 1998; Lange and Croteau, 1999; Eisenreich et al., 2001). Then 1-deoxy-D-xylulose 5-phosphate is converted to 2-C-methyl-D-erythritol 4-phosphate (MEP) via the reductase DXR (Takahashi et al., 1998). MEP is then converted to 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MECPP) in three sequential steps. MEC is converted to 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate by the enzyme 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate synthase (HDS), which is then reduced to 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate via the enzyme 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate reductase (HDR). 1-hydroxy-2-methyl-2-(E)-butenyl 4-diphosphate is finally converted to IPP with the enzyme HMBPP reductase. IPP is then isomerized to DMAPP (Lichtenthaler, 1999).

In both cytosolic and plastidic compartments, IPP and DMAPP are used by prenyltransferases to produce prenyldiphosphates, the universal precursors of all isoprenoids. A head to tail condensation of IPP and DMAPP results in the formation of geranyldiphosphate (GPP) or neryl diphosphate (NPP), the *trans* and *cis* prenyldiphosphate precursor of monoterpenes, respectively. Sequential addition of one more molecule of IPP by farnesyl-diphosphate synthase results in the formation of all-*trans* or *cis-trans* farnesyl diphosphate (*ee*-FPP and *zz*-FPP), the precursors of sesquiterpenes. For the synthesis of the precursor of

diterpenes, geranylgeranyldiphosphatase (GGDP), the addition of one more molecule of IPP is required that is catalyzed by the prenyltransferase geranylgeranyldiphosphate synthase. In *C. creticus* subsp. *creticus* two genes that encode active GGDP synthases, *CcGGDPS1* and *CcGGDPS2*, were cloned and functionally characterized (Pateraki and Kanellis, 2008), as also the DXR cDNA from trichomes (Pateraki and Kanellis, 2004).

The prenyldiphosphate precursors are converted to the basic terpene skeletons through the activity of a class of enzymes called terpene synthases. In the lower orders of terpenes these enzymes are further categorized into monoterpene, sesquiterpene and diterpene synthases. The basic skeletons are often further processed by a variety of enzymes including acyltransferases, hydroxylases and dehydrogenases to produce the thousands of different terpenes encountered in nature. There are both cyclic and acyclic terpenes whose exact structure is determined by the specific activity of terpene synthases and the subsequent modifying enzymes. Terpene synthases are classified into two groups based on their catalytic mechanisms: class I and class II reviewed in Chen et al. (2011). In class I enzymes, the prenyldiphosphate precursor is ionized resulting in the formation of a carbocation intermediate, which undergoes cyclizations, hydride shifts and other rearrangements that result in the formation of the basic terpene skeletons of the products. Depending on these rearrangements, some class I terpene synthases specifically give rise to a single product, while others are characterized as multiproduct enzymes. This class of terpene synthases includes primarily monoterpene and sesquiterpene synthases and some diterpene synthases. Class II terpene synthases catalyze a protonation-induced cyclization of the prenyldiphosphate precursor. The reaction is terminated either via deprotonation or nucleophile capture. Characteristic enzyme of this category is the copalyldiphosphate synthase responsible for the synthesis of copalyldiphosphate, the intermediate precursor of *ent*-kaurene and several labdane-type diterpenes.

Most diterpenes are cyclic and their carbon-rings are formed by two different mechanisms. One is similar to that of class II monoterpene and sesquiterpene synthases. Examples of such reactions are those that generate macrocyclic diterpenes such as casbene and taxadiene (Dueber et al., 1978; Koepp et al., 1995). The second mechanism includes the biosynthesis of a cyclic diphosphate intermediate via class II terpene synthases, the final product being achieved through the class I terpene synthase.

Labdane-type diterpenes represent a distinct class of terpenoids with a characteristic basic bicyclic skeleton connected to an additional six-carbon chain (cyclic or acyclic) that may or may not contains an oxygen atom. Initial data on labdane-type diterpene biosynthesis came from studies on the biosynthesis of *ent*-kaurene, the diterpenoid precursor of gibberellins, and other labdane-type diterpenes that do not contain oxygen in their skeleton. *Ent*-kaurene biosynthesis involves a two-step reaction, first the cyclization of GGDP to *ent*-copalyldiphosphate (*ent*-CPP) by copalyldiphosphate synthase (CPS), and then its conversion to *ent*-kaurene by *ent*-kaurene synthase (KS). Copalyldiphosphate synthase performs a protonation-initiated cyclization (class II) while *ent*-kaurene synthase performs an ionization-initiated cyclization of CPP (class I) (Sakamoto et al., 2004).

The biosynthesis of labdane-type diterpenes that function as phytoalexins in rice (*Oryza sativa*) involves *ent*-CPP or *syn*-CPP as intermediates (Otomo et al., 2004; Prisic et al., 2004; Xu et al., 2004). These phosphorylated intermediates are then further cyclized to the final diterpene products namely oryzalexins, momilactones, and phytocassanes (Nemoto et al., 2004; Otomo et al., 2004; Wilderman et al., 2004; Kanno et al., 2006). All angiosperm terpene synthases may have evolved from the gymnosperm group of bifunctional class II/I terpene synthases producing labdane-related diterpenes. Characteristic examples are: the abietadiene synthase from *Abies grandis* (Vogel et al., 1996), the levopimaradiene synthase from *Ginkgo biloba* (Schepmann et al., 2001), and the levopimaradiene/abietadiene from *Picea abies* (Martin et al., 2004). These bifunctional enzymes catalyze both reactions, first cyclizing GGDP to CPP and then converting CPP to the final tricyclic terpene, that can be further modified by additional enzymes like hydroxylases, methyltransferases, etc. (Ro et al., 2005; Hamberger and Bohlmann, 2006; Ro and Bohlmann, 2006).

The biosynthesis of oxygen-containing labdane-type diterpenes was only recently unraveled. First, it was shown that protein extracts from *Nicotiana glutinosa* and *N. tabacum* trichomes that contain labdane-type diterpenes such as abienol, labdenediol, and sclareol, could be converted to all the above oxygen-containing diterpenes *in vitro* with externally supplied GGDP (Guo et al., 1994; Guo and Wagner, 1995). This observation led to the hypothesis that their synthesis involved a copal-8-ol diphosphate intermediate, the synthesis of which is initiated by protonation of the terminal double bond of GGDP, and the formation of a bicyclic carbocation followed by capture of a hydroxyl anion (Guo and Wagner, 1995). Indeed, it was later shown that this type of enzyme exists.

C. creticus copal-8-ol diphosphate synthase (CLS) is a type II terpene synthase expressed in the trichomes of *C. creticus*, which catalyzes the formation of the copal-8-ol diphosphate from GGDP (Falara et al., 2010) (Figure 2). Copal-8-ol diphosphate can then be cyclized to labda-13-en-8 α ,15-diol, labda-14-en-8,13-diol (sclareol), manoyl-oxide and 13-*epi*-manoyl-oxide. A similar pathway employing copal-8-ol diphosphate was shown to operate in the trichomes of *N. tabacum* for the biosynthesis of Z-abienol (Sallaud et al., 2012).

In *Salvia sclarea*, a class I diTPS (SsSS) has been characterized that transforms the copal-8-ol diphosphate intermediate into sclareol (Caniard et al., 2012; Schalk et al., 2012). In *Coleus forskohlii*, CfTPS2 catalyzes the synthesis of copal-8-ol diphosphate, which is then utilized by CfTPS3 to its stereospecifically form (13R)-manoyl oxide, the precursor of the highly complex labdane diterpene forskolin (Pateraki et al., 2014). A different phosphorylated intermediate, labd-7,13-dien-15-yl diphosphate, is hypothetically converted to labd-7,13-dien-15-ol and then further processed to produce its derivative labd-7,13-dien-15-yl acetate (Figure 2). In fact, we have recently functionally characterized in *E. coli* and in yeast a gene from *C. creticus* that converts GGDP to labd-7,13-dien-15-yl diphosphate (Papaefthimiou et al., 2013). Taken together with the function of CcCLS, we suggest that for each labdane-type diterpene class in *Cistus*, a unique class II synthase may be required. Recently, a bifunctional

labdane-type diterpene synthase producing labd-7,13-dien-15-ol has been characterized from the gymnosperm *Selaginella moellendorffii* (Mafu et al., 2011).

PHENYLPROPANOIDS

Phenylpropanoid compounds are abundant in the plant kingdom, either providing plants with a valuable defensive arsenal against pathogens, herbivores, and environmental stressors or facilitating the plants reproductive machinery. In addition, these molecules have important applications in the fragrance industry and in medicine. The precursor of all plant phenylpropanoids is *trans*-cinnamic acid, derived from the amino acid phenylalanine, the first enzymatic step being catalyzed by phenylalanine ammonia-lyase (PAL) leading to the synthesis of *p*-coumaroyl-CoA, the substrate of more complex aromatic phenylpropanoids (Vogt, 2010). Numerous chemicals are classified as plant phenolic secondary metabolites, which are further categorized within several distinct groups based on their basic skeleton as found in simple phenols (C₆). The more complex phenylpropanoid compounds include catechol (C₆)_n, phenolic acids (C₆-C₁), phenylacetic acids (C₆-C₂), coumarins (C₆-C₃), lignans (C₆-C₃)₂, lignins (C₆-C₃)_n, naphthoquinones (C₆-C₄), xanthenes (C₆-C₁-C₆), stilbenes (C₆-C₂-C₆), flavonoids (anthocyanins, isoflavonoids, flavones, flavanes, proanthocyanidins, etc.) (C₆-C₃-C₆) and tannins (C₆-C₃-C₆)_n. The biosynthetic pathways of several groups belonging to the very diverse phenylpropanoid compounds have been studied in several model plants, including *Arabidopsis thaliana*, *Medicago truncatula*, *Nicotiana benthamiana*, *Oryza sativa* (Vogt, 2010). In *Cistus*, phenolic compounds have been the target of many chemical, biological and taxonomic studies, already discussed in earlier sections of this review and summarized in Table S3. On the basis of these studies, a general pathway of the phenylpropanoid biosynthesis in *Cistus* can be drawn (Figure S2). Several genes involved in the plant phenylpropanoid pathway have been isolated from various plant genera, while some important enzymes were further characterized and novel pathways were discovered (Cheynier et al., 2013). However, the biosynthetic pathway leading to the production of phenylpropanoids and phenolic compounds has not been yet studied in *Cistus*.

GENOMIC ANALYSES AND BIOTECHNOLOGICAL APPROACHES

GENOMIC ANALYSES

Cistus plants are very rich sources of secondary metabolites, which make it hard to isolate quality nucleic acids in sufficient amounts. A protocol for the efficient isolation of high quality DNA and RNA from *C. creticus* subsp. *creticus* was published (Pateraki and Kanellis, 2004). This paved the way for a series of molecular studies in *C. creticus* subsp. *creticus* aiming for the elucidation of the terpenoid biosynthetic pathway. In view of the valuable properties and possible future exploitation of these natural products it was deemed necessary to study their biosynthesis and its regulation at the molecular level. In this direction, sequence based and functional genomic approaches were initiated. Initial work characterized the expression of genes coding for *CcHMG*R in the MVA pathway, *CcDXS* and *CcDXR* in the

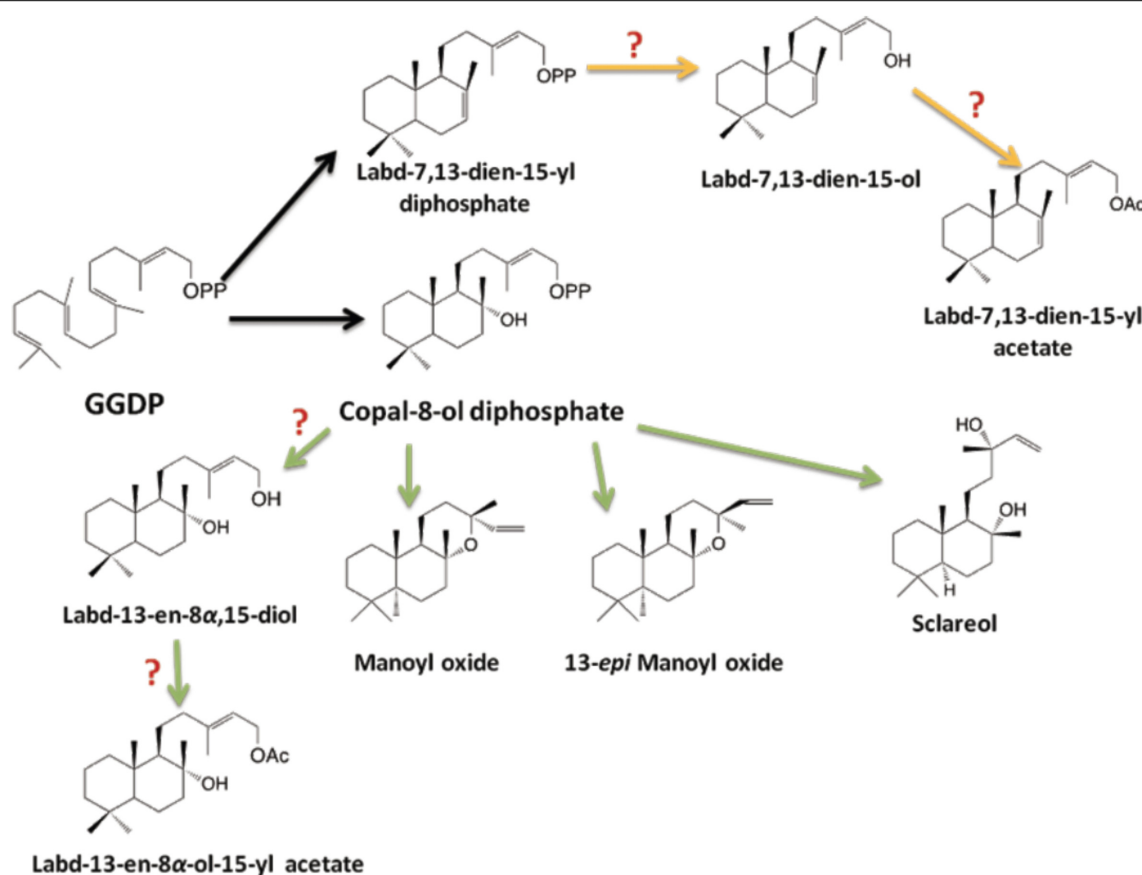


FIGURE 2 | Proposed pathway to labdane-type diterpenes predominant in *C. creticus* resin. A protonation-initiated cyclization catalyzed by CcCLS converts GGDP to the stable bicyclic intermediate copal-8-ol diphosphate. A second ionization-initiated cyclization of copal-8-ol diphosphate results in the formation of manoyl oxide isomers, while labd-13-en-8 α ,15-diol could be formed either by phosphatase activity or type A diterpene synthase activity. Similarly a

different phosphorylated intermediate, labd-7,13-dien-15-yl diphosphate is hypothesized to be converted to labd-7,13-dien-15-ol and then further processed to produce its derivative labd-7,13-dien-15-yl acetate. The acetylated products need the function of novel acetyltransferase(s). Black arrows indicate the biosynthetic steps that involve already characterized enzymes. Question marks indicate pathway steps that have not yet been characterized.

MEP pathway, and followed by the characterization of the two *CcGGDPS1* and *CcGGDPS2* (Pateraki and Kanellis, 2008). This work suggested that leaf trichomes are very active biosynthetically for terpenoids, and the pathway is regulated at the transcriptional level. Moreover, *CcHMGR* and *CcDXS* transcripts (the rate-limiting steps of the isoprenoids' pathways) increase during mechanical wounding or upon treatment with stress hormones such as JA and SA, which possibly reflects an increased need of the plant tissues for the corresponding metabolites (Pateraki and Kanellis, 2010).

The first genes isolated and functionally characterized from *C. creticus* subsp. *creticus* were *CcGGDPS1* and 2 coding for synthases of geranyl-geranyl diphosphate, the precursor of all diterpenes (Pateraki and Kanellis, 2008). Heterologous expression in *Saccharomyces cerevisiae* revealed that these full-length cDNAs possessed GGDPs enzyme activity. Gene and protein expression investigations proposed that this enzyme is developmentally and tissue-regulated showing maximum expression in trichomes and smallest leaves (0.5–1.0 cm).

Next, in order to search for putative terpene synthases, an EST library was built using RNA extracted from trichomes

isolated from young leaves (Falara et al., 2008). This was decided on the basis of chemical profiling which showed that young leaf trichomes were richer in labdane-type diterpenes compared to mature ones. The subsequent EST analysis that was conducted produced 2022 clones (Falara et al., 2008; <http://www.ests-pharm.web.auth.gr/ests.php>). Functional annotation of the 2022 expressed sequence tags (ESTs) from the trichome cDNA library, based on homology to *A. thaliana* genes, showed that 8% of the putative identified sequences belonged to secondary metabolism pathways mainly in flavonoid and terpenoid biosynthesis. Custom DNA microarrays assembled with 1248 individual clones from the cDNA library enabled transcriptome comparisons between trichomes and trichome-free tissues. Verification of the DNA microarrays data by RT-PCR pinpointed a germacrene B synthase (*CcGerB*) as a trichome specific gene (Falara et al., 2008). Further, the isolation of the promoter of this gene was achieved (Saramourtsi, 2013).

The full-length cDNA of copal-8-ol diphosphate diterpene synthase (*CcCLS*) was functionally characterized from *C. creticus*, elucidating the novel first step in the labdane-type diterpenes biosynthetic pathway not only in *Cistus* but in all angiosperms

(Falara et al., 2010, Figure 2). Gene expression analysis revealed that *CcCLS* is preferentially expressed in trichomes, with higher transcript levels measured in the glandular trichomes of young leaves compared to fully expanded leaves. Interestingly, *CcCLS* transcript levels increased after mechanical wounding used to simulate herbivore attack. Chemical analyses revealed that labdane-type diterpene production in general followed a similar pattern, with higher concentrations in trichomes of young leaves and increased accumulation upon wounding, indicating that increased diterpene biosynthesis is related to the plant's defense mechanisms. Application of New Generation Sequencing (NGS) in *C. creticus* trichomes RNA resulted in a total of 385,143 contig sequences (114,239 unigenes) with a mean length of 207 nucleotides. Among those, 2000 unigenes were related to the biosynthesis, transport and catabolism of secondary metabolites. Twenty partial sequences were phylogenetically related to putative diterpene synthases (Papaefthimiou et al., 2013). Among those, two diterpene synthases have already been functionally characterized (see Biosynthetic Pathway for Terpenes Including Labdane-Type Diterpenes).

Another protein isolated and characterized from *C. creticus* was the key transcriptional regulator *TRANSPARENT TESTA GLABRA1* (*CcTTG1*) (Ioannidi, 2009). In *Arabidopsis*, *TTG1* is involved in trichome differentiation, in the regulation of flavonoids and in seed mucilage production (Walker et al., 1999). The glabrous phenotype of the loss of function *A. thaliana ttg1* mutants was restored upon transformation with *CcTTG1*. The gene was also able to restore the seed coat pigmentation defect of the mutant. The yeast two-hybrid screen resulted in the identification of seven *CcTTG1* interactors. Four of them were further analyzed using the yeast-two-hybrid system. Three protein-protein interactions were tested *in planta*, using a transient expression system in tobacco epidermal cells. The analysis was based on the Bi-molecular Fluorescent Complementation (BiFC) and verified the interaction of *CcTTG1* with two *Squamosa* promoter Binding Proteins (SBP). Also the interactions were detected in the nucleus of the tobacco epidermal cells. This work reported the first protein-protein interaction of the SBP family of transcription factors and a novel interaction of the *CcTTG1* protein (Ioannidi, 2009).

Other genetic data available for *Cistus* is restricted to genes used as molecular markers in taxonomic and phylogenetic studies. Several studies include the isolation and use of partial sequences from a variety of commonly used molecular markers in order to achieve the delimitation of *Cistus* species. These include the nuclear (*nucGS*, *ITS*) and plastid (*trnL-trnF*, *trnK-matK*, *trnS-trnG*, *rbcl*) DNA sequences, the *trn-F* and *RPL32-TRNL* sequences of *cpDNA* (Falchi et al., 2009) RNA polymerase subunits (Pawluczyk et al., 2012), as well as genetic markers (*ISSR*—PCR amplification) (Paolini et al., 2009).

IN VITRO CULTIVATION OF CISTUS

The first *in vitro* cultivation of *Cistus* was reported in 1991 (M'Kada et al., 1991) on stem nodal segments for the multiplication of *Cistus X purpureus* Lam, using MS medium (Murashige and Skoog, 1962) supplemented with cytokinins. Later, a protocol was established for *in vitro* propagation of *Helianthemum*

almeriense (Cistaceae), using MS with plant growth elicitors for shoot generation (Morte and Honrubia, 1992). Soon after, an improved protocol for *in vitro* propagation was established with high concentrations of growth elicitors in MS medium leading to successful propagation of six rock-rose species (*C. albidus* L., *C. clusii* Dunal, *C. ladanifer* L., *C. laurifolius* L., *C. psilosepalus* L., and *C. salviifolius* L) (Iriondo et al., 1995). *In vitro* propagation of *C. creticus* was successfully established by three separate approaches. In the first approach, a combination of growth elicitors was used on shoots proliferation and on callus regeneration (Pela et al., 2000). In the second approach, shoot formation was obtained 30 days after the first subculture in WPM. The segments were rooted, after supplementing the medium with IBA (0.98–3.94 μ M) or NAA (0.1–0.5 μ M), and zeatin (0.2–0.5 mg l⁻¹) was used for callus induction (Zygomala et al., 2003). In the third and most recent work, studying the micropropagation of *C. creticus* (Madesis et al., 2011), rapid proliferation of shoot-tips was achieved using MS, supplemented with growth elicitors, and after 4 weeks, shoots were transferred to MS for rooting or further development. In order to achieve rooting, shoots longer than 1 cm were used and cultured on MS without growth regulators. *In vitro* proliferation of *Cistus* has also been studied in *C. clusii*, with satisfactory results (Ruta and Morone-Fortunato, 2010). In conclusion, methodologies are in place for *in vitro* cultivation of *Cistus* plants.

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Does groundwater protection in Europe require new EU-wide environmental quality standards?

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The European Groundwater Directive could be improved by limiting the scopes of the Annexes I and II to the manmade and natural substances, respectively, and by defining a common monitoring protocol. The changes in the European land use patterns, in particular the urban sprawl phenomena, obscure the distinction between the point and diffuse sources of contamination. In the future more importance will be given to the household contamination. Moreover, the agricultural environment could be used for developing new conceptual models related to the pharmaceuticals.

Keywords: groundwater monitoring, GWD, pesticide, nitrates, OWC, conceptual modeling, annex I, annex II

INTRODUCTION

Groundwater is a source of fresh water for human consumption, irrigation and ecosystem needs, and its protection is a key environmental objective. Its pollution by anthropogenic activities is a threat to human health and wellbeing and to dependent ecosystems. The European Union has promoted several directives for protecting groundwater from pollution and deterioration (Quevauviller, 2006): the Water Framework Directive (WFD, 2000/60/EC), the Groundwater Directive (GWD, 2006/118/EC), the Nitrates Directive (91/676/EEC), the Landfill Directive (99/31/EC) and the Directive on Environmental Quality Standards in the Field of Water Policy (EQSD, 2008/105/EC), recently amended by Directive 2013/39/EU.

The Water Framework Directive (WFD), and in particular the related Groundwater Directive on the protection of groundwater against pollution and deterioration, establish criteria for: (1) the assessment of good chemical status (2) the identification and reversal of environmentally significant pollutant trends; (3) preventing or limiting inputs of pollutants into groundwater.

In 2009, the EU funded a 5 year project named GENESIS (VII Framework Programme). The project, coordinated by the Norwegian Institute for Agricultural and Environmental Research (Bioforsk), involved 25 partners from 17 countries. The objective of the GENESIS was to integrate pre-existing and new scientific knowledge into the development of methods, concepts, and tools for better management of groundwater resources. One of the project's main tasks was to provide suggestions for the revision of the Groundwater Directive.

During the project experts with various background have been working together to develop the approach of a better integrated groundwater system management. Impacts of land use and climate change have been integrated with the conceptual models of groundwater systems emphasizing on the role of Groundwater Dependent Ecosystems (GDEs). Achievement of this goal has been possible by twining expertise and knowledge gained from case studies on different hydrogeological, land use, and socio-economic settings. As an outcome a range of methods has been presented for the future management of aquifers.

In July 2013, the European Commission opened a public consultation to provide input to the first review of the Directive Annexes. This consultation was intended for stakeholders, experts and practitioners in public authorities involved or interested in the implementation of the GWD. The expertise gained during the course of the GENESIS project implementation allowed for providing input to two of the four topics identified by the public consultation, in particular on updating the list of substances regulated at EU and national levels. The project addressed also the knowledge gaps related to the occurrence and risk assessment for different groundwater contaminants, including the emerging contaminants.

DISCUSSION

THE ANNEX I SHOULD INCLUDE THE MANMADE SUBSTANCES AND THE ANNEX II THE NATURAL ONES

Annex I of the GWD contains Europe-wide environmental quality standards (EQS) for two types of pollutants: nitrates and

active substances and relevant metabolites in pesticides. Annex II contains a minimum list of pollutants and indicators to be provided by Member States for the establishment of threshold values and identifies information on those pollutants and indicators which have been indicated as contributing to the characterization of bodies or groups of bodies of groundwater as being at risk. Article 10 of the GWD obliges the Commission to review Annexes I and II of the Directive every 6 years and to come forward with legislative proposals, if appropriate.

The Annex I set the EQSs according to the regulations being in force at that time; in particular the nitrate standard was established based on epidemiological studies (WHO, 2011) and the pesticides standard took into account the detection limit of the analytical equipment available at the beginning of the 90 s.

For naturally occurring substances the variability of the groundwater systems across the EU does not allow for the determination of common threshold values for the whole European territory, similarly like for nitrates and pesticides, which are regarded as substances of anthropogenic origin. For the naturally occurring substances, threshold values are based on the determination of the natural groundwater quality expressed as Natural Background Levels (NBLs). However, NBLs are highly variable depending on the geological environmental (e.g., karstic aquifers, presence of geothermal fields, mineral deposits, etc) and the location of the aquifer (e.g., coastal aquifers). Additionally, the existence of pristine areas where NBLs can be estimated is questionable. For these reasons the substances that require the NBL assessment are reported in Annex II.

In the Annex II list two man made substances are reported (Tetrachloroethylene and Trichloroethylene), probably because these substances were considered as originating from the point and not the diffuse contamination sources. In our opinion the scope of the Annex I should be limited to manmade substances for which it is possible to establish EQS and the Annex II should be reserved for the substances that require assessing of the NBLs.

A COMMON MONITORING PROTOCOL IS REQUIRED

In 2010, the European Commission reviewed the threshold values established by Member States (European Commission, 2010a). Up to date, threshold values (TVs) have been reported across EU for 158 pollutants/indicators, either synthetic or naturally occurring (European Commission, 2009, 2010a). The range of values and the methodologies used show great diversity across Member States. Moreover for some pollutants/indicators included in the Annex II not all Member States (MS) have established the TVs (Table 1). Thus, those propositions cannot be considered as comparable and consistent. For this reason a unique methodology for TVs assessment should be proposed by the EU and adopted by Member States. Based on the type of substances the methodology should account for different geological settings resulting in different NBLs in groundwaters, and the various Environmental Quality Standards (EQS) which are set taking into account the various receptors (aquatic or terrestrial ecosystem or “groundwater itself”) and the various legitimate uses of groundwater. The methodology should also call for a common monitoring protocol across EU member states, in order maximize reliability of monitoring results.

Table 1 | Number of member states (MS) that have established threshold values (TV) for the substances included in annex II of the GWD (European Commission, 2010a).

Pollutants/indicators	Number of MS (EU27) that established TV
Ammonium	20
Ammonium (as nitrogen)	2
Arsenic	21
Cadmium	19
Chloride	22
Conductivity	14
Lead	20
Mercury	18
Sulphate	21
Sum of Trichloroethylene and Tetrachloroethylene	10
Tetrachloroethylene	10
Trichloroethylene	10

THE DISTINCTION BETWEEN THE POINT AND DIFFUSE SOURCE OF CONTAMINATION IS QUESTIONABLE

The inclusion of contaminants coming from household or from industrial drivers in the Annex I is subjected to intense criticism on the basis that they are the point sources. However, there is a strong evidence that the aquifers contaminated by industrial and household urban contaminants at European scale can no longer be considered as affected only by point-type sources because the urban areas become intermixed with agricultural and natural areas, as a consequence of the effects of urban sprawl; and a high number and density of potential sources of pollution and already polluted hotspots, gives to this type of contamination a “diffuse source” pattern and no more a “point source” one.

More than a quarter of the European territory is nowadays directly affected by urban land use (European Environmental Agency, 2006); urban areas are becoming the typical landscape of many parts of Europe or, at least, the typical landscape of areas inhabited by the majority of Europeans. The urban sprawl is advancing at a very high rate and the urban areas in 2013 are much more extended all around the continent, with respect to 2000, when the Water Framework Directive was adopted. Urban sprawl can be considered as one of the most significant land-use transformation affecting Europe and is now rightly regarded as one of the major challenges facing urban Europe today (European Environmental Agency, 2006). In 2006, in the same year when the Groundwater Directive was adopted, the Environmental European Agency reported that the urban-area expansion rate of several eastern and western European countries had increased by over three times with respect to the growth rate of their population. Many recently sprawled zones are located in alluvial and/or coastal areas or within mountainous and/or foothill basins, frequently in the recharge areas of the aquifers historically used for drinking water supply. In some cases, these areas are inside or close to protection areas of wells or springs.

In the Northern Italy, one of the most populated area in Europe characterized by a typical mixed urban-agricultural landscape, Scalenghe et al. (2011) studied the influence of 150 years of

land use on anthropogenic and natural carbon stocks in Emilia-Romagna region, that represents 16% of the 71,000 km² of the Po River watershed. At the present time it is home to about 18 million inhabitants, with an average population density of 300 people / km². This region produces 40% of the Italian gross domestic product, one-third of the national industrial and arable production and has half of the Italian animal husbandry. The Human Footprint, which globally is 28, in this area scores higher than 50 (Sanderson et al., 2003). Scalenghe et al. (2011) used the amount of carbon contained in concrete as an indicator of urbanization demonstrating that it has increased in their study area from about 2.5 Tg in the 1954 to 7.8 Tg in 2003. The maps produced in their study show that urban areas constitute a diffuse point source. The Corine Land Cover (CLC2000) reports that artificial surfaces cover 6% of the basin area. The river Po management plan (Autorità di bacino del fiume Po, 2010) shows that in the Po valley 1.3 waste water treatment plants per 100 km² and 4.6 industrial point discharges per 100 km² are present. Above the aquifer the density of industrial discharges rises to 6.8. This change in the land use causes the increase of non-point sources defined as improperly managed construction sites (Frumkin, 2002), former contaminated industrial sites under remediation (Callender and Rice, 2000; Van Metre and Mahler, 2010) and the sewage system network that is worldwide known as one of the most important source of contamination in urban areas (Nolan et al., 2002; Masetti et al., 2007). The new residential areas are also entering on peripheral pre-contaminated sites associated with industrial activities or landfills (Nijenhuis et al., 2013).

THE DPSIR FRAMEWORK REQUIRES BETTER CHARACTERIZATION OF THE HOUSEHOLD CONTAMINATION

The European Union identified the Driver, Pressure, State, Impact, Response (DPSIR) framework as the analytical framework for reaching the goal of achieving good groundwater status (European Commission, 2003). DPSIR has been used for identifying impacts and pressures on groundwater resources in order to attain the goal of good status across Europe. Under this approach, a "Driver" is an anthropogenic activity that can affect the environment; a "Pressure" is the direct effect of a Driver; and an "Impact" is the environmental effect of a Pressure. "State" is the environmental condition resulting from the Pressure and 'Responses' are the measures taken to improve the State. DPSIR coupled with the groundwater characteristics has been used to develop conceptual models and to establish local monitoring programmes. Conceptual models are a means of describing and optionally quantifying systems, processes and their interactions (European Commission, 2010b) and are developed to different incremental degrees of complexity.

The Common Implementation Strategy identified households, industry, agriculture, forestry, mines and quarries, dump and storage sites, water abstraction and flow enhancement as the Drivers that can affect the status of groundwater.

The analysis of the pollutants and of the indicators within DPSIR reveals that pressures coming from agriculture are monitored by at least three classes of compounds (trace elements, pesticides, and nutrients). Contaminants coming from households and industry are represented in Annex II only by trace

elements and by two substances belonging to crude oil contaminants. The new amended Groundwater Directive should require the monitoring of more indicators representative of industrial and household contamination. Annex 1 should include at least an indicator representative of the Industry Driver and an indicator representative of Household driver.

TETRACHLOROETHYLENE AND TRICHLOROETHYLENE CAN BE CANDIDATE INDICATORS OF THE URBAN SPRAWL

Urban sprawl phenomenon is a source of new groundwater pollutants coming from industrial and domestic sources: chemicals not previously included in national or international monitoring programmes (Reemtsma et al., 2008), and contaminants that have long occupied attention, are gaining new notoriety with the discovery of new aspects of their occurrence, fate or effects (Daughton, 2004). All those substances are labeled as emerging pollutants and they encompass antiseptics; abuse drugs, antioxidants; corrosion inhibitors, fragrances, insect repellents, flame retardants, gas propellants, plasticizers, pharmaceuticals, solvents, stain repellents, sterols, stimulants, sunscreens and surfactants (Balderacchi et al., 2013; Fatta-Kassinos and Michael, 2013).

Among those Emerging Contaminants, Tetrachloroethylene or Perchloroethylene (PCE) and Trichloroethylene (TCE) can be considered an excellent indicators of the groundwater pollution by multi-source diffuse type urban pollution because their environmental behavior and toxicity have been thoroughly studied. We suggest removing those compound from annex II and including them in the EQS list of the improved annex I for three reasons:

Chlorinated solvents, like PCE and TCE, are the most prevalent organic contaminants found in groundwater (Stroo et al., 2003)

Chlorinated aliphatic solvents are a large family of compounds that are widely used in several industries all over Europe. Due to improper use and disposal practices, these solvents (mainly PCE and TCE) are common organic contaminants of soil and groundwater (Kao and Lei, 2000; Rivett and Feenstra, 2005). They have a peculiar ability to infiltrate rapidly into the subsurface, causing soil and groundwater pollution (Kueper et al., 2003; Cortés et al., 2011). There are several thousand of PCE and TCE impacted sites throughout North America, continental Europe and other industrialized areas of the world (Hunkeler and Aravena, 2000; Kueper et al., 2003; Parker et al., 2003; Cortés et al., 2011). Many of these sites are affected by releases of DNAPL that took place since the first half of the 20th century (Kueper et al., 2003).

PCE and TCE have features that favor their persistence and areal diffusion in groundwater

They are typically mobile and recalcitrant (Guilbeaut et al., 2005; Rivett and Feenstra, 2005) and they originate, at the source, as immiscible liquids: at many PCE and TCE spill sites, residual amounts of these compounds persist in a pure liquid phase, commonly referred to as dense non-aqueous-phase liquids (DNAPLs), within pore spaces or fractures (U.S. EPA., 1992; Feenstra et al., 1996; Kao and Lei, 2000). Ground water flowing through the DNAPL zones dissolves them, generating plumes that

commonly achieve exceptionally large sizes (Schwille, 1984, 1988; Mackay and Cherry, 1989). PCE and TCE may decompose into even more hazardous pollutants. Where the unfavorable environmental conditions occur, the persistence of these compounds in the hydrogeological system can lead to reductive microbial dechlorination of PCE and TCE to dichloroethene (DCE) isomers and vinyl chloride (VC) (Vogel and McCarty, 1985; Barrio-Lage et al., 1987), which are well known carcinogenic compounds. Moreover, they can cross low permeability geological layers that do not act as protective barriers as they do for groundwater. Solvent DNAPLs can penetrate into or through most types of aquitards, even those with very low bulk hydraulic conductivity, due to naturally occurring preferential pathways (Parker et al., 2004). Resultingly, not only phreatic aquifers but also the confined ones are highly vulnerable toward PCE and TCE contamination. They can closely interact with low-permeability deposits, especially those having a significant organic matter content, where they can be trapped and subsequently released into the aquifers (Chapman et al., 2012) in such a manner that these deposits become a secondary source of pollution persisting for times estimated at up to hundreds of years (Chapman and Parker, 2005). Consequently, low permeability deposits can release to groundwater significant amounts of pollutants resulting through such back diffusion in their dangerous concentrations, (Parker et al., 2004). The occurrence and the persistence of these processes corroborate the opinion that they should be considered as representative of non-point pollution sources too. Remediation of these pollutants can be very difficult to conduct. Experience from the past 20 years has demonstrated that DNAPL sites are difficult to investigate and challenging to remediate. Interest in strategies for source removal has increased since the mid-1990s. Innovative technologies were developed and marketed to overcome the perceived technical impracticability of source treatment (Kueper et al., 2003). Therefore, given that it is often not possible to locate and remove the residual PCE and TCE, remediation should focus on preventing further migration of dissolved contamination (Kao and Lei, 2000).

PCE and TCE have been detected as ubiquitous in extensive areas of European aquifers

Many urban areas all around Europe observe a widespread pattern of PCE and TCE occurrence in groundwater. Very often plumes, coming from single point-sources, intermingle and coalesce between each other revealing a diffuse pattern of pollution. Chlorinated solvents typically cause a persistent environmental contamination, which, in most cases, started decades ago (Cortés et al., 2011).

In their studies in the UK, Longstaff et al. (1992) found that low-level contamination is often widespread, with “hotspots” of higher concentrations. “Hotspots” of higher level groundwater contamination are often associated with sites where solvents were used, although obvious sources of solvent contamination could not be found for all “hotspots.” Moreover, several studies from over the whole Europe report cases in which PCE and TCE contamination is widespread on a large area, even if it originated from point sources (Italy: Cavellero et al., 1985; CNIC, 2010; Nijenhuis et al., 2013; Netherlands: Zoetmann et al., 1981;

Germany: McCann and Appleton, 1993; Spain: Cortés et al., 2011; UK: Rivett et al., 1990). A recent investigation, conducted in Campania region nearby Naples (Southern Italy), has put in evidence that, inside a mainly agricultural-used land, due to the occurrence of multi-sources illegal dumps, PCE is the most widespread contaminant (CNIC, 2010).

SHOULD WE CONSIDER OTHER EMERGING SUBSTANCES?

Until now, water quality legislation has not systematically dealt with emerging pollutants for several reasons, including a lack of knowledge on contaminant sources and pathways, the properties and effects of substances and analytical detection techniques. Recently, scientific interest is given to organics waste water contaminants (OWCs), which are all the substances that reach waste water treatment plant, from veterinary antibiotics to industrial contaminants. Critical opinions against the monitoring of other emerging pollutants arise because of the high cost of the monitoring and the poor knowledge about those substances. However the study of those “emerging substances” is required for the progress of the science and for giving to the citizen a more safe environment. Because of this safety demand and the lack of conceptual models for emerging pollutants in groundwater, policy-makers and scientists are cooperating for the creation of an initial groundwater emerging pollutant priority list. The directive 2013/39/EU introduces a watch list of substances that will be completed in 2014 and that now includes Diclofenac, 17-beta-estradiol and 17-alpha-ethinylestradiol.

The classification of emerging pollutants according to the source of contamination or the toxicity is effective and pragmatic but does not consider the interaction of the substances with the soil that in the case of groundwater contamination is crucial and therefore the development of the conceptual model is expected. The selection of those substances has to be carried out considering two main drivers pressures:

1. The diffuse contamination coming from the agriculture driver. In this case, the hydrogeological conceptual models are already available and therefore biogeochemical models can be developed easily. Veterinary pharmaceuticals are good candidates for the inclusion in the monitoring plans because they are pharmaceuticals widely used: Kools et al. (2008) estimated that more than 5000 t of antibiotics and 5 t of hormones are employed in the European meat production. The substances are generally known and not patented because they have been on the market since long time. They contaminate the manure and slurry and are distributed during the agricultural production assuming diffuse contamination path.
2. The quasi “point source” contamination coming from household. In view of the scarce knowledge of the contamination pathways, the first attempts will have to focus on simplified pathways: from households to WWTPs to rivers and to groundwater; from biosolids and graywater to soils and groundwater. It is also required to collect information on monitoring point construction details, hydrological settings, aquifer type, understanding of recharge sources and patterns, local groundwater flow patterns and regimes, abstraction impacts, residence times and groundwater age distributions

(European Commission, 2007). Environmental tracers can provide valuable information on natural attenuation of dissolved organic contaminants in groundwater systems. Isotope tracers constitute an important and widely used tool in studies on the environmental fate of biodegradable organic contaminants (Hunkeler et al., 2008; Aelion et al., 2009), providing evidence for and insights into mechanisms of microbial decomposition.

CONCLUSIONS

Nowadays more than a quarter of the European territory has been directly affected by urban land use and the changes in the society and in particular the urban sprawl phenomena is also changing the current conceptual models based on the DPSIR framework. The scientific progress is providing evidence, that contamination pathways, previously linked to point sources are now considered as originating from diffuse sources. New environmental quality standards have to be defined in addition to nitrates and pesticides for protecting the groundwater quality at the European scale. PCE and TCE are increasingly destined to be considered as indicators of a widespread source of contamination, rather than a point-source phenomenon and therefore they can be candidate to be the best indicator of the groundwater pollution by multi-source diffuse type urban pollution of groundwater.

Other emerging pollutants are attracting the interest of scientists and policy makers. The monitoring of those substances is a necessary step in the creation of conceptual models for the improvement of the DPSIR framework. The veterinarian pharmaceuticals framed within the driver agriculture and the pressures associated to this driver can be an excellent starting point because diffuse contamination agricultural conceptual models are already developed and these models can help to understand the behavior of pharmaceuticals into the environment.

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Bimodular high temperature planar oxygen gas sensor

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A bimodular planar O₂ sensor was fabricated using NiO nanoparticles (NPs) thin film coated yttria-stabilized zirconia (YSZ) substrate. The thin film was prepared by radio frequency (r.f.) magnetron sputtering of NiO on YSZ substrate, followed by high temperature sintering. The surface morphology of NiO NPs film was characterized by atomic force microscope (AFM) and scanning electron microscope (SEM). X-ray diffraction (XRD) patterns of NiO NPs thin film before and after high temperature O₂ sensing demonstrated that the sensing material possesses a good chemical and structure stability. The oxygen detection experiments were performed at 500, 600, and 800°C using the as-prepared bimodular O₂ sensor under both potentiometric and resistance modules. For the potentiometric module, a linear relationship between electromotive force (EMF) output of the sensor and the logarithm of O₂ concentration was observed at each operating temperature, following the Nernst law. For the resistance module, the logarithm of electrical conductivity was proportional to the logarithm of oxygen concentration at each operating temperature, in good agreement with literature report. In addition, this bimodular sensor shows sensitive, reproducible and reversible response to oxygen under both sensing modules. Integration of two sensing modules into one sensor could greatly enrich the information output and would open a new venue in the development of high temperature gas sensors.

Keywords: NiO nanoparticles, high temperature, oxygen sensing, bimodular, potentiometric, resistance

INTRODUCTION

Every year, a huge amount of liquid and solid fuels are consumed through the combustion process (Ramamoorthy et al., 2003). Therefore, *in-situ*, real-time monitoring the composition of combustion gases is important for improving combustion efficiency and reducing the emission of pollutants such as CO, NO_x, etc. (Miura et al., 2000; Fergus, 2007a,b, 2008; Zhuiykov and Miura, 2007). According to a U.S. Department of Energy report, harsh environment sensors, if successfully employed, are predicted to save 0.25 quadrillion BTU/year of energy across all energy-consuming industries (Miura et al., 2000; Akbar et al., 2006). Therefore, there is an urgent need to design fast, sensitive, selective, rugged, and cost-effective high-temperature gas sensors for power/fuel systems. In the past decades, various gas sensors have been developed for monitoring combustion process (Szabo et al., 2003; Zhang et al., 2007; Liu and Lei, 2013). Among them, solid-electrolyte potentiometric, amperometric, and semiconductor oxide sensors have been extensively studied for high temperature environment.

In particular, high temperature potentiometric sensors have played a pivotal role in pollution control through automobile engine management (Azad et al., 1992; Fergus, 2007a). For the equilibrium potentiometric oxygen sensor, the most direct type uses a solid electrolyte that conducts an ion of the species to be detected, such as an oxygen-ion conductor. Equilibrium potential

measurements on solid electrolyte-electrode cells enable oxygen measurement via the Nernst law and the sensor voltage signal typically varies with oxygen partial pressure logarithmically. Disadvantages of such potentiometric sensors include unavailable sensing materials for certain gas species and the requirement of a gas-tight seal between reference and working electrode. Recently, mixed potential gas sensor has attracted much attention and been proposed as an alternative to classical potentiometric sensors due to its stability, sensitivity, and selectivity at high temperatures (Fergus, 2007a; Morata et al., 2008; Plashnitsa et al., 2008). When the sensor is exposed to oxygen, sensor response (electromotive force, EMF) is controlled by the difference in kinetics of redox reactions of oxygen at electrode/electrolyte/gas interface (triple phase boundary, TPB) (Li and Kale, 2007; Sekhar et al., 2010). For the mixed potential type sensors, chemical reactions tend toward equilibrium with increasing temperature, so a sensor response that depends on kinetically limited response leads to decreased signals with the increase of temperature. Consequently, mixed potential type sensors are not favorable for application in very high temperature environment.

High temperature sensors can also be operated in the amperometric mode, in which the electrode reaction is caused by an applied potential and the limiting current, as controlled by diffusion through a small orifice or porous media, is measured (Fergus, 2007a). The mode based on stabilized zirconia exhibits

several advantages to detect gaseous compounds like O_2 and NO_x under harsh environments (Reinhardt et al., 2000). Linear relations between the measured current and gas concentrations can be achieved. Although, many of fuel economy vehicles use amperometric sensors, physical modification of the diffusion barrier of the sensor is possible to cause problems (Ramamoorthy et al., 2003). In addition, sensor selectivity is a challenge for amperometric sensors, since any species containing the mobile ion can contribute to the current and thus interfere with the sensor response.

The resistor-type sensor based on semiconducting metal oxide is another commonly used group of high temperature sensors which possess many advantages, such as simple configuration, easy fabrication and cost effectiveness. In resistor type oxygen sensor, a voltage is applied across the electrodes (Liu et al., 2012a) and the response of the sensor toward oxygen can be measured through changes in electrical conductance arising from alteration of the defect chemistry by chemisorption of oxygen and/or reactions between oxygen gas and sensing material (Ramamoorthy et al., 2003; Bartic et al., 2007). The bulk defect phenomena are related to oxygen ion vacancies, metal ion interstitials and vacancies, electrons and holes. The change in conductance of the material upon exposure to oxygen can be explained by the change in concentration of oxygen species in the bulk, and is a reflection of the defect structure. The predominant defects in oxide semiconductors are oxygen vacancies and their associated free charge carriers. N- and P-type semiconductors have inverse directions of conductivity's change upon the interaction with oxygen, which is a very important fact for their application (Korotcenkov, 2007). For n-type semiconductor its conductivity drops upon exposure to oxygen, whereas for p-type oxide it rises.

As an important p-type semiconductor material, NiO has been receiving great research interest due to its wide band gap property, superior thermal and chemical stability. Consequently, NiO becomes a promising material in many applications such as giant magnetoresistance sensor, gas sensors, electrochemical display devices and conducting transparent electrodes (Hotovy et al., 2004; Lee et al., 2009; Gupta et al., 2012).

In this research, a bimodular oxygen sensor, including potentiometric module and resistance module, was developed through a planar sensor platform using NiO nanoparticles (NPs) thin film, which was r.f. magnetron sputter-coated on YSZ surface followed by sintering at 1100°C . The surface morphology of NiO NPs thin film was characterized by scanning electron microscope (SEM), atomic force microscope (AFM). X-ray diffraction (XRD) was applied to characterize its crystal structure and thermal stability of NiO NPs thin film before and after high temperature gas sensing. Furthermore, the oxygen sensing properties of the bimodular oxygen sensor operated at 500, 600, and 800°C was investigated under potentiometric module as well as resistance module, both of which showed sensitive, reproducible and reversible response to oxygen. The sensing mechanisms under both sensing modules were also discussed. These features demonstrate that the combination of two sensing modules into one sensor could greatly enrich the information output and has great potential in the development of novel high temperature gas sensors.

MATERIALS AND METHODS

FABRICATION OF BIMODULAR SENSOR DEVICE

An yttria-stabilized (8.0%) zirconia plate (Coating & Crystal Technology Inc., USA) with a dimension of 8 mm (length) $\times$ 8 mm (width) $\times$ 0.5 mm (thickness) was used for fabrication of the bimodular planar high temperature gas sensor device. Before loading into sputter coating chamber, the YSZ plate was chemically cleaned in order to remove dust and organic contamination (if any). Nickel oxide NPs thin films with a thickness of about 100 nm were sputtered on one side of YSZ plate by r.f. magnetron sputtering technique. Platinum (Pt) paste was then printed on the other side of YSZ plate, serving as the reference electrode in the potentiometric mode sensor. The sensor device was sintered at 1100°C for 3 h in air to increase its high temperature stability. The configuration of bimodular sensor is shown in Figure 1.

CHARACTERIZATION OF NiO NANOPARTICLES THIN FILM

The three-dimensional morphology of sputter-coated NiO NPs thin film was examined by AFM (Asylum Research MFP-3d). A JEOL 6335F field-emission SEM at 10 kV was employed to observe the morphology of sensing electrode. A Bruker XRD instrument D2 Phase with a $\text{Cu K}\alpha$ X-ray source ($\lambda_\alpha = 1.54 \text{ \AA}$) was used to analyze the crystalline phase of NiO NPs thin film before and after high temperature oxygen sensing. The data were collected in the 2θ range of $10\text{--}90^\circ$ with a scanning rate of $12^\circ/\text{min}$.

HIGH TEMPERATURE GAS TESTING SYSTEM

The NiO-YSZ substrate was connected with four Pt wires to realize bimodular monitoring—two Pt wires on NiO NPs thin film side used for resistance detection module and the other two Pt wires (one on NiO side and the other on Pt paste side) used for potentiometric module (Figure 1). The four Pt wires were pressed on NiO-YSZ plate by two alumina plates through mechanical force and thus built up good contact and connection with NiO-YSZ plate at its both sides. The device was then placed in a temperature-controllable furnace which connected with a mass flow controller. The two pairs of fixed Pt wires were then connected to two CHI 660D Electrochemical workstation

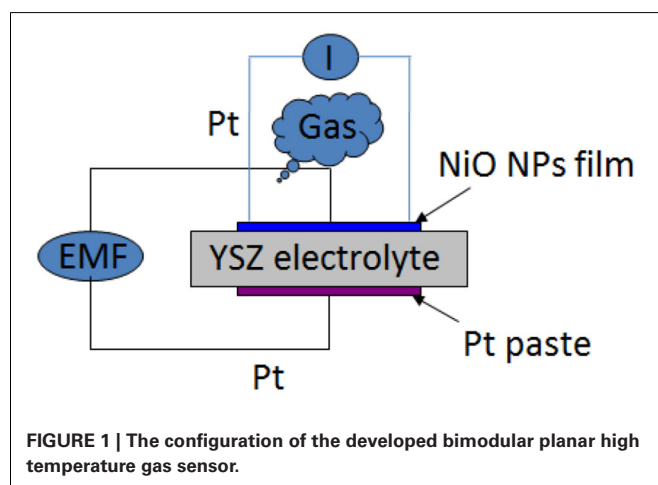


FIGURE 1 | The configuration of the developed bimodular planar high temperature gas sensor.

(CH Instrument, USA) through Ni/Cr alloy wires for continuous recording of *in-situ* EMF change and resistance change of the sensing device under different oxygen concentrations and operating temperatures.

Oxygen sensing experiments were performed with a total gas flow of 1.5 L/min at different O₂ concentrations ranging from 100 to 800 ppm and at various operating temperatures (500, 600, and 800°C). Nitrogen gas was used as the carrying gas. In a typical O₂ sensing experiment, the sensor was first exposed to high purity nitrogen for 2 h until the baseline was stable, and then exposed to O₂/N₂ for 6 min, followed by high purity N₂ to recover the sensor for 18 min (one cycle). For each oxygen concentration, two exposure/recovery cycles were conducted. The sensor circuit was subjected to 1 V DC voltage during the resistance sensing module, while for potentiometric sensing module, the EMF measurements were carried out between both sides of YSZ planar sensor with NiO NPs thin film side connected to the working electrode and Pt paste side connected to the reference electrode.

RESULTS AND DISCUSSION

CHARACTERIZATION OF NiO FILM

As shown in **Figure 2**, the AFM topography of the as-deposited NiO NPs thin film revealed that the sputter-coated NiO film, after sintering at 1100°C, consists of numerous densely-packed NPs which are in good agreement with observation in SEM image (**Figure 3**). The grain size of NiO was 78 ± 13 nm according to SEM characterization. Such highly porous thin film could provide large surface for gas interaction and minimize gas diffusion resistance, thus favoring subsequent high temperature gas sensing.

The composition and crystal structure of the as-deposited NiO NP thin film were further characterized by XRD. The XRD patterns of the NiO NPs film before and after high temperature oxygen sensing are shown in **Figure 4**. The formation of face-centered cubic NiO is confirmed by the diffraction peaks at 2θ values of 37.3, 43.3, and 79.4°, corresponding to (111), (200), and (222) crystal planes, respectively (**Figure 4A**). Other peaks can be assigned to YSZ substrate (**Figure 4C**). The XRD pattern (**Figure 4A**) matches the standard spectrum of JCPDS 4-835 (**Figure 4D**) except from two perspectives. Firstly, the peak corresponding to (220) is not obvious, which was consistent with

other studies (Chen et al., 2005; Jang et al., 2008). Jang et al. found that there is no (220) peak for NiO after sputter-coating even with heat treatment (Jang et al., 2008). Chen et al. reported that texture coefficient (220) decreased with increasing of temperature (Chen et al., 2005). Secondly, the intensity of (111) diffraction peak is much larger than that of (200), which is different from that of JCPDS-4-835. It is known that metal oxide samples prepared in

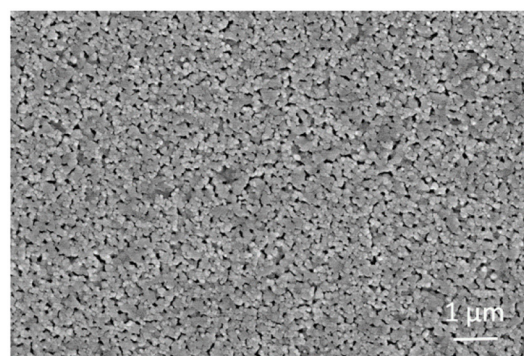


FIGURE 3 | A typical SEM image of the NiO nanoparticle thin film on YSZ plate after sintering.

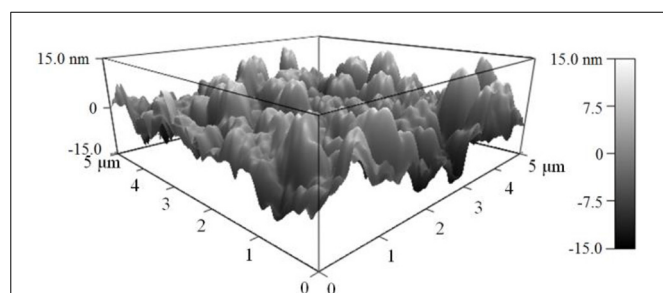


FIGURE 2 | AFM image of the surface ($5 \times 5 \mu\text{m}$) of NiO nanoparticles thin film on YSZ plate after sintering at 1100°C.

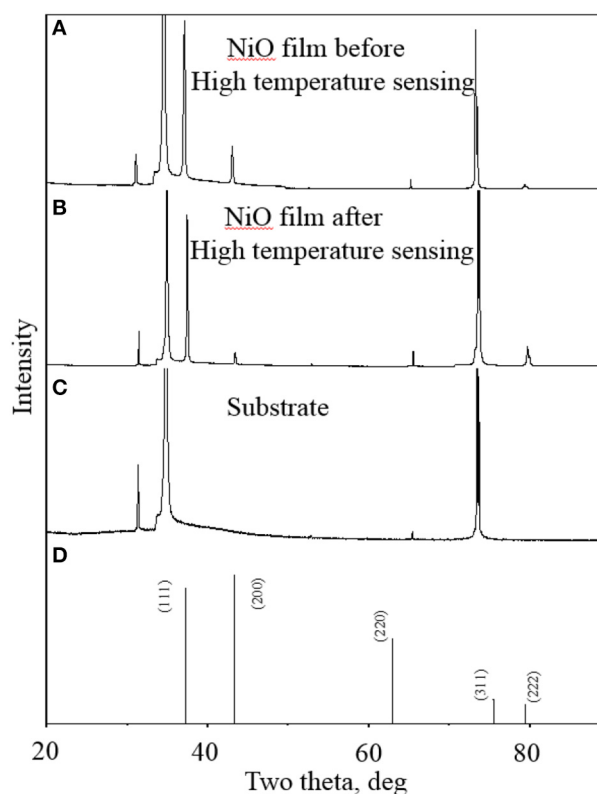


FIGURE 4 | XRD patterns of NiO nanoparticle thin films (sintered at 1100°C) before (A) and after (B) high temperature oxygen sensing experiments; (C) XRD pattern of YSZ substrate; and (D) XRD pattern of standard pattern of NiO.

the oxide-sputtering mode showed a strong (111) diffraction peak and its intensity increased with increasing annealing temperature (Hotovy et al., 2004). Therefore, such intensity difference can be attributed to the sintering process under a high temperature of 1100°C. In order to analyze its thermal stability in high temperature sensing processes, the XRD pattern of NiO NPs thin film after high temperature oxygen sensing experiments was also investigated and presented in **Figure 4B**. One can see that the XRD pattern shows no peak shift compared to that before high temperature gas sensing, suggesting the superior chemical and thermal stability of NiO NPs thin film in high temperature sensing environment. Such good chemical and thermal stability endows the as-prepared NiO NPs thin film capability for high temperature O₂ sensing.

HIGH TEMPERATURE OXYGEN SENSING UNDER POTENTIOMETRIC MODULE

The oxygen sensing property of the NiO NPs thin film was examined at operating temperatures of 500, 600, and 800°C, respectively, under potentiometric module. **Figure 5** presents the typical EMF values of the sensor device as a function of time upon periodic exposure to different concentrations of O₂ at different operating temperatures. One can see that at the operating

temperatures of 500 and 600°C, the EMF values decreases rapidly upon the exposure to oxygen and displays a concentration-dependent behavior. The sensor response can be rapidly recovered using high purity N₂ at both operating temperatures. The sensitivity to oxygen could majorly arise from the combined effects of the difference in the kinetics of oxygen reduction at TPB, catalytic activity of the electrode as a function of the concentration in the electrode, and the microstructure of the sensing electrode relative to the Pt electrode (Li and Kale, 2007). These results were in good agreement with literature report of mixed-type potentiometric oxygen gas sensor (Li and Kale, 2007). However, baseline drifting was observed at an operating temperature of 500°C, similar phenomena has also been observed by other groups (Morata et al., 2008), which suggests slower kinetics for the recovery of sensor response at lower operating temperatures. However, at 800°C, the response pattern is slightly different from those at 500°C and 600°C. Especially, the EMF value of the sensor device first recovers to a value higher than the initial EMF value and then gradually declines to the initial EMF level, indicating that a more complex mechanism may involve at higher working temperature. The dissimilar oxygen reaction on two electrodes and diffusion may play a larger role at 800°C with respect to the NiO, YSZ, and Pt surfaces in this case.

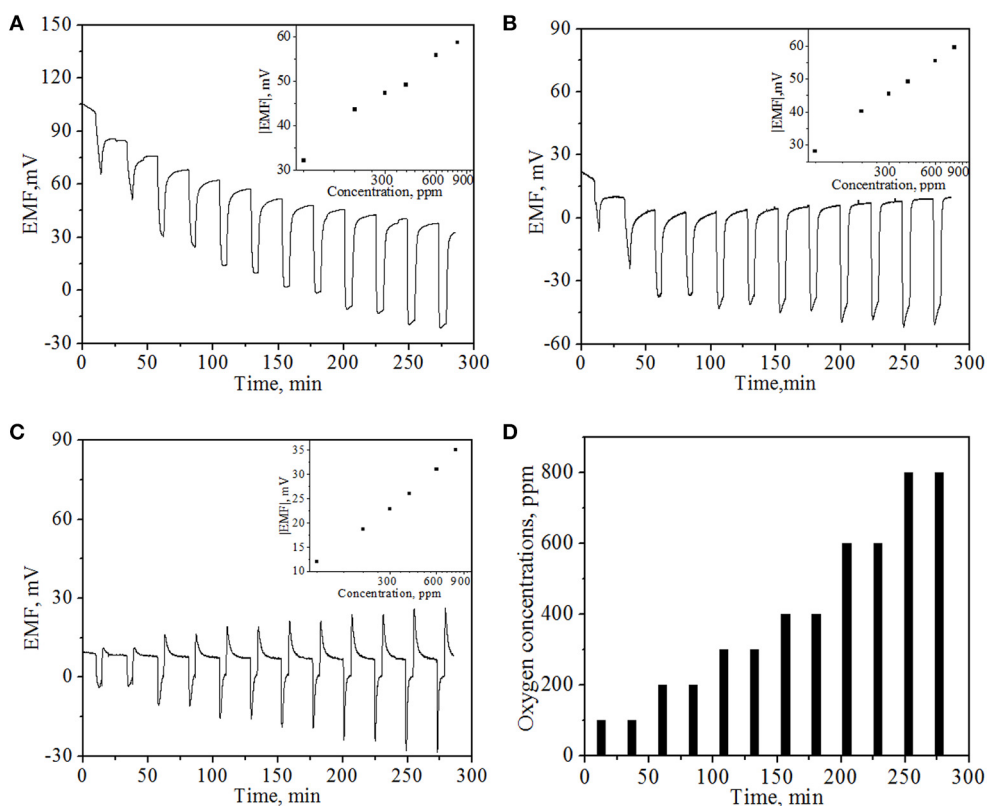


FIGURE 5 | In-situ EMF values of potentiometric mode sensor at different operating temperatures: (A) 500°C; (B) 600°C; (C) 800°C with various oxygen concentrations. Insets show the corresponding average

EMF values of the sensor at different O₂ concentrations. **(D)** The pattern of supplied oxygen with various concentrations in the oxygen gas sensing experiment.

The NiO NPs thin film based potentiometric sensor can be described in the form of electrochemical cell as shown below:

In the base gas, N_2 , $NiO|YSZ|Pt, N_2$
 In the sample gas, O_2+N_2 , $NiO|YSZ|Pt, O_2+N_2$

The oxygen electrochemical reaction ($O_2 + 4e^- \rightleftharpoons 2O^{2-}$) is equilibrating. However, the reactions take place at different rates on the dissimilar electrodes, and thus a potential difference is generated, which leads to a measurable EMF variation. Besides electrochemical reaction mechanism, Bartolomeo et al. proposed adsorption mechanism (Di Bartolomeo et al., 2004), in which the variation of EMF is consistent with the changes in resistance induced by chemisorption of oxidizing or reducing gas on n-type or p-type semiconductors related to electrocatalytic activity. NiO is a p-type semiconductor at high temperature. When NiO is exposed to oxygen at the operating temperature, the resistance of NiO decreases and thus leads to the decrease of EMF value.

The insets of **Figure 5** present the plots of average EMF value vs. O_2 concentrations at corresponding operating temperatures. It can be observed that EMF value of the sensor device exhibits a linear dependence on the logarithm of oxygen concentrations, as expected from well-established Nernst equation (Ramamoorthy et al., 2003; Moos et al., 2009). In addition, it can be observed from **Figure 5** that in general, the sensor response to the same oxygen concentration follows the decline trend with the increase of temperature. For example, the EMF values were 47.3, 45.5, and 22.8 mV for 300 ppm O_2 concentration at 500, 600, and 800°C, respectively. This phenomenon was compatible with previous reports (Di Bartolomeo et al., 2004; Fergus, 2007a; Plashnitsa et al., 2008) and also can be well-explained by aforementioned adsorption mechanism (Di Bartolomeo et al., 2004). In general, similar mixed potential difference response tends to decrease with increasing temperature because of following reasons. Chemical reactions tend toward equilibrium with increasing temperatures, thus a sensor response heavily relies on kinetically limited response. The increase of temperature would lead to a faster kinetics for gas redox reaction on both electrodes, resulting in a reduction of potential difference between them. Therefore, mixed potential type sensor has one drawback that cannot be applied in very high temperature environment, which greatly limits its broader applications as high temperature gas sensors.

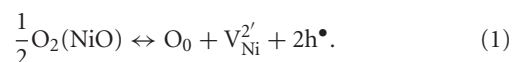
HIGH TEMPERATURE OXYGEN SENSING UNDER RESISTANCE MODULE

The O_2 sensing properties of the sensor device were further examined at operating temperatures of 500, 600, and 800°C, respectively, under resistance module (the resistance of NiO film). The applied bias in the sensing experiments was 1 V. The current in the sensor was continuously measured and the electric resistance of the sensor was calculated by applying Ohm's Law ($R = V/I$). R_0/R , where R is the real-time sensor resistance with change of gas concentrations and R_0 is the initial sensor resistance in N_2 , were employed as the sensor response. **Figure 6** represents typical electrical responses of the sensor device as a function of time upon periodic exposure to gaseous oxygen balanced in high purity N_2 . Upon exposure to oxygen, the NiO NPs thin film

shows reproducible, sensitive and concentration-dependent resistance change to oxygen, suggesting that NiO NPs thin film is a good sensing material for resistance-based oxygen detection at high temperature. By purging with nitrogen, the response of sensor can be recovered, indicating the quick desorption of oxygen molecules from the NiO NPs and the good reproducibility of NiO NPs thin film based oxygen sensor. The good performance and response/recovery can be attributed to the highly porous structure of sensing material and nanoscale size of NiO, in which porous structure allows the free access of oxygen molecules to NiO with reduced diffusion resistance while nanoscale size allows fast chemisorption and desorption of gas molecules (Liu et al., 2012a). In addition, no obvious baseline drifting was observed for repeated operation, indicating the good stability.

At fixed operating temperatures, the sensing response (R_0/R) increases with oxygen concentration from 100 to 800 ppm. For example, at an operating temperature of 600°C, the response increases from 4.03 to 5.89 when O_2 concentrations increase from 200 to 800 ppm. At fixed oxygen concentration (e.g., 300 ppm oxygen), the sensing response increases from 3.33 to 4.57 to 5.34 when the temperature increases from 500°C to 600°C to 800°C, respectively, which is different from the observation for previous potentiometric module. This result implies that resistance sensing module may have greater potential to be applied in higher temperature environment compared to potentiometric module. Combining two detection modes into one sensor, one can realize a wide operating temperature window and also greatly enrich the information output.

At high temperatures, NiO exhibits a p-type semiconductor behavior. First, NiO can be readily made slightly oxygen-rich and thus its crystal must have a population of point defects (Tilley, 2011). This can be considered as a reaction of oxygen gas with stoichiometric NiO, assuming that extra oxygen extends the crystal by adding extra oxygen sites. As reported earlier (Tilley, 2011), the following defect reaction can occur:



The creation of each cation vacancy is accompanied by the creation of a hole. For NiO, ionic crystal structure is assumed, and then a p-type behavior would be expected. Consequently, with the increase of oxygen concentrations, the above equation shifts to the right and generates more holes, thus leading to an increase of electrical conductivity, in good agreement with the experimental results. On the other hand, removal of O_2 could shift the equation to the left side, and then bring the conductivity back to the original level and regenerate the sensor.

A relationship between the oxygen partial pressure and the electrical conductivity of an oxide (Ramamoorthy et al., 2003; Liu et al., 2012b) can be further represented by

$$\sigma = A \exp(-E_a/kT) P_{O_2}^m \quad (2)$$

where σ is the electrical conductivity, A is a constant, E_a is the activation energy for conduction, P_{O_2} is the oxygen partial pressure, and m is a parameter determined by both the type of the carrier (n or p) and the defects (e.g., oxygen vacancy) in the

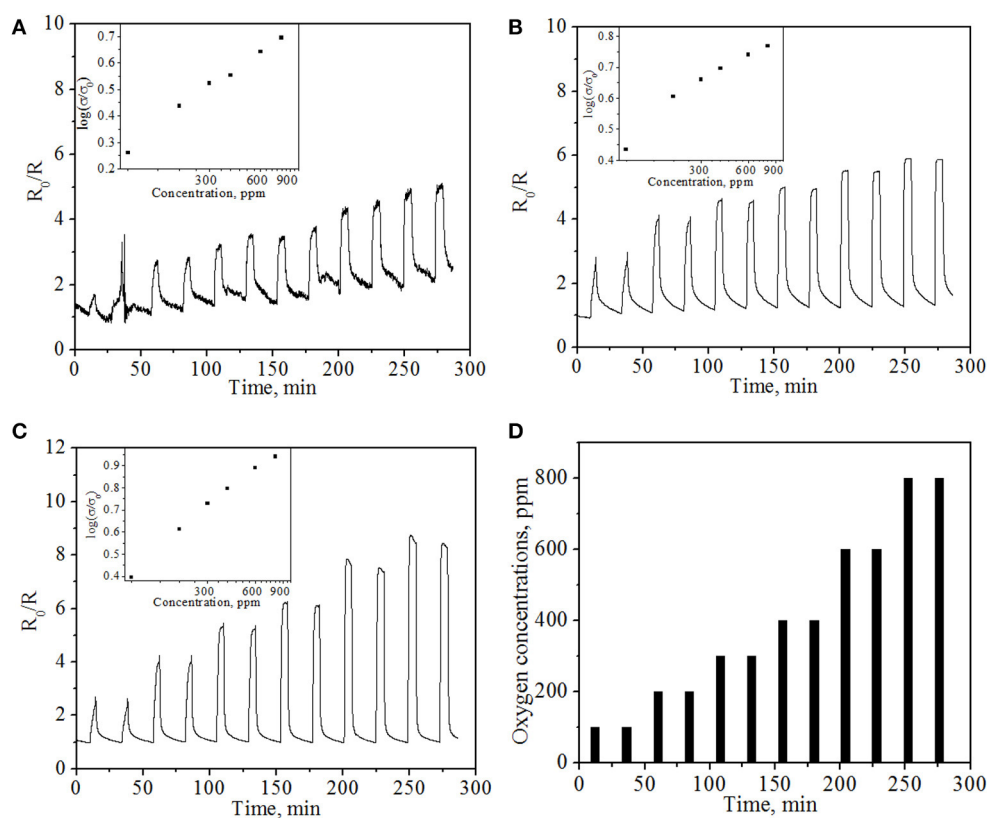


FIGURE 6 | In-situ sensing response of resistance type sensor at different operating temperatures: (A) 500°C; (B) 600°C; (C) 800°C with various oxygen concentrations. Insets show the average of normalized

electrical conductivity of the sensor vs. oxygen concentration. (D) The pattern of supplied oxygen with various concentrations in the oxygen gas sensing experiment.

semiconductor. For p-type oxide, m is a positive value. The normalized electrical conductivity of NiO NPs thin film vs. oxygen concentration at different temperatures is shown in the insets of **Figure 6**. At a fixed operating temperature, the normalized conductivity is proportional to the logarithm of oxygen concentration, which was consistent with the aforementioned equation as well as the experimental results. However, compared to the theoretical value $m = 1/6$ to $1/4$ in $\sigma \propto P(O_2)^m$ (Stubican and Carinci, 1998; Birks et al., 2006), the obtained m values for NiO NPs thin film are 0.42, 0.27, and 0.54 at 500, 600, and 800°C, respectively. The value of m at 600°C is very close to the theoretical value $1/4$, while the values at 500 and 800°C are slightly larger than the predicted value. Similar phenomenon has been observed in literature, in which m value was reported to be close to 0.5 due to the potential contribution of hole concentration on the surface (Palombiari, 2003). The observed high m values in this study may be ascribed to the influence of donor-acceptor coupling, high hole concentration on the surface, significant contribution of ionic conductivity, and/or grain boundary diffusion associated with nanoscale NiO (Liu et al., 2012b). It is also possible that YSZ electrolyte plays a role in m value, since YSZ is a good ionic conductor at elevated temperatures. Typically, the higher the m value, the higher the oxygen response of the sensor. Therefore, the high oxygen response of NiO NPs film obtained in this study indicates that NiO NPs film is a good candidate in the

construction of bimodular gas sensor for the detection of oxygen in high temperature environment. According to Equation 2, the calculated activation energy is 12.4 ± 1.3 kJ/mole, which is also in good agreement with reported values of $10.4 \sim 13.8$ kJ/mole and 12.5 kJ/mole (Hakim et al., 2009; Guziewicz et al., 2011).

CONCLUSIONS

In summary, NiO NPs thin film with good thermal stability was successfully coated on YSZ substrate by r.f. magnetron sputter-coating followed by sintering at 1100°C. A novel bimodular planar O_2 sensor based on the as-prepared NiO/YSZ was then fabricated and applied for high temperature oxygen detection under two modules—potentiometric and resistance. Both potentiometric module and resistance module of the developed bimodular sensor exhibit sensitive, reproducible and reversible response to oxygen. The sensing mechanisms for both modules were also discussed. Integration of two sensing modules into one sensor could greatly enrich the information output and would open a new venue in high temperature gas sensor development.

AUTHOR CONTRIBUTIONS

Xiangcheng Sun performed the experiments and analyzed the data. Xiangcheng Sun and Yu Lei conceived and designed the experiments, performed the data analyses and wrote the manuscript. Yixin Liu contributed to the resistance module

experimental design and explanation. Haiyong Gao participated in NiO film preparation and AFM characterization. Pu-Xian Gao and Yu Lei coordinated and financed the project. All the authors commented on the manuscript.

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Toward a comprehensive and realistic risk evaluation of engineered nanomaterials in the urban water system

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The European COoperation in Science and Technology (COST) Action ES1205 on the transfer of Engineered Nano materials from wastewater Treatment and stormwater to Rivers (ENTER) aims to create and to maintain a trans European network among scientists. This perspective article delivers a brief overview on the *status quo* at the beginning of the project by addressing the following aspects on engineered nano materials (ENMs) in the urban systems: (1) ENMs that need to be considered on a European level; (2) uncertainties on production-volume estimations; (3) fate of selected ENMs during waste water transport and treatment; (4) analytical strategies for ENM analysis; (5) ecotoxicity of ENMs, and (6) future needs. These six step stones deliver the derivation of the position of the ES1205 network at the beginning of the projects runtime, by defining six fundamental aspects that should be considered in future discussions on risk evaluation of ENMs in urban water systems.

Keywords: engineered nanomaterials, water treatment, wastewater, urban water system, fate of nanomaterials, nanomaterials toxicity, transformation of nanomaterials, risk assessment on nanomaterials

INTRODUCTION

The COST Action ES1205 ENTER was launched in June 2013 (<http://www.es1205.eu> and http://www.cost.eu/domains_actions/essem/Actions/ES1205). In close cooperation with the NORMAN network working group 4 (<http://www.norman-network.net/?q=node/54>) the Action aims to create and maintain a lasting pan European network among scientists to gain a better understanding of the role of the urban water systems controlling the release of ENMs to the aquatic and associated environments (e.g., sewage sludge or wetland soils, and sediments). By May 2014 28 COST and none COST countries as well as more than 140 scientists joined the network. Key questions are which and what amounts of ENMs are released, how persistent are they and to what extent do they cause *in situ* toxicity? The scope of this perspective article aims to sum up the status and lack of current knowledge in answering the above mentioned questions. Out of several sources-sink scenarios (Figure 1) the COST Action ES1205 will prioritize the potential pathways in the following order:

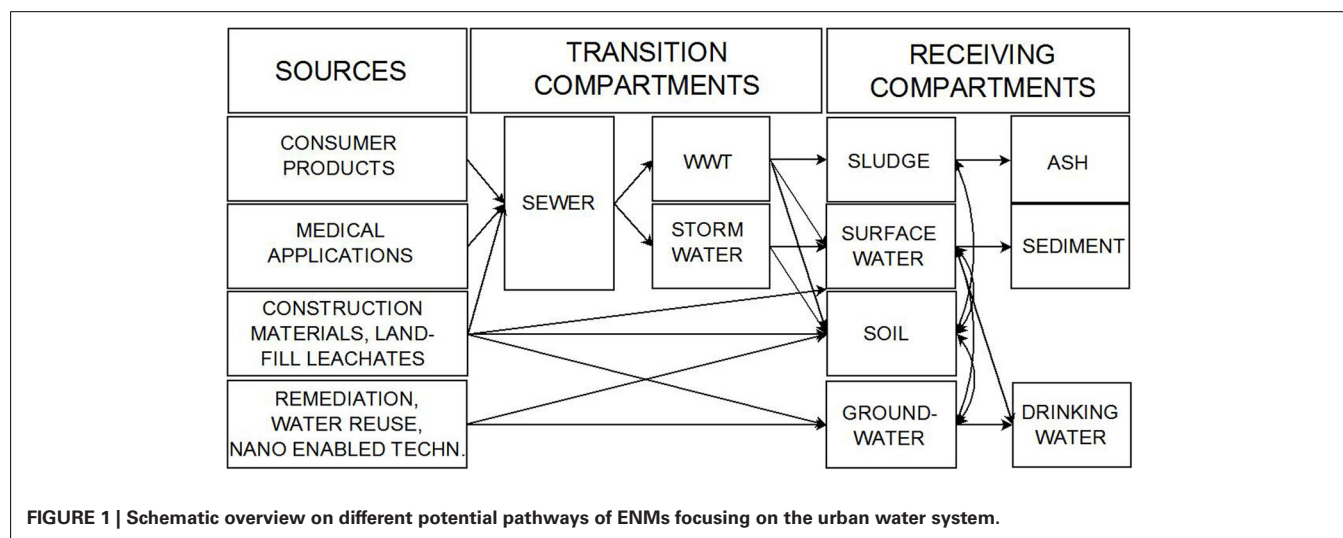
- (1) wastewater → wastewater treatment (WWT) → surface water, sediments;
- (2) wastewater → sewage sludge → (incineration plant) ash disposal, soil conditioner, fertilizer → soil;
- (3) wastewater → stormwater → surface water, sediments, stormwater treatment systems (e.g., constructed wetlands), and

- (4) landfill leachate → treatment → surface water, sediments.

As soon as the step from fundamental research toward considerations on the environmental relevance of ENMs is undertaken, the production volumes of ENMs have to be evaluated and their mobility in the environment has to be assessed. Different release, exposure and fate scenarios have to be evaluated, depending on the application and life cycle of different types of ENMs. Consequently, a connection between the ENM applications and potential sources into urban water systems has to be established.

A thorough understanding of potential pathways and linkage of transformation and retention processes of ENMs in different compartments is highly needed to enable a valid risk evaluation. A fundamental requirement for a mechanistic understanding of ENM behavior in addition to ecotoxicity studies, are sufficiently applicable analytical tools for complex matrices. This, in connection with fate and toxicity studies enables a comprehensive and realistic risk evaluation of ENMs in urban water systems.

This perspective article highlights and critically discusses briefly the *status quo* of scientific findings regarding ENMs at the beginning of the COST Action. Given the fact that silver nanoparticles (Ag-NPs) are strongly discussed in science and the public (but of less relevance from an application and transformation point of view) literature was cited (where possible) for clarity reasons.



CONSIDERED ENMs ON AN EUROPEAN LEVEL

ENMs cover a heterogeneous range of different materials, including: inorganic (non-metallic), carbon based, metal, and organic nanomaterials. Due to this and the fact that a uniform statutory demanded product labeling, indicating whether “nano” is “inside” a product or not, is mostly voluntary, market information on ENM production volumes and types are not readily available.

Hence, the European Commission (EC) composed a working paper on types and uses of nanomaterials (EU Commission, 2012) providing an overview on ENMs commercially in use. On the basis of this working paper it turned out that two materials in the nano range dominate the market: carbon black (9.6 million t/a) and amorphous silica (1.5 million t/a). Further ENMs produced in significant amounts are: aluminium oxide (200 kt/a), barium titanate (15 kt/a), titanium dioxide (10 kt/a), cerium oxide (10 kt/a), zinc oxide (8 kt/a), and carbon materials (bandwidth 0.1–1 kt/a). Silver has been listed with minor priority (20 t/a). Main fields of applications of the above listed ENMs are: reinforcing agents in rubber goods (mainly carbon black, amorphous silica) followed by applications in electronics (e.g., fine abrasives, capacitors), cosmetics, biomedical applications (e.g., gold for diagnostics, silver in textiles), paints (e.g., pigments) and surface-coatings (e.g., scratch-resistance). Nevertheless, assessments on market information of ENMs need to be taken with caution.

As a further source of information a number of web-based platforms exist listing ENMs being commercially used. This data is linked to “day-to-day use goods” (e.g., at www.nanotechproject.org/ or www.nanowerk.com). But again, this information needs to be interpreted with caution. As nanotechnology is a rapidly developing field opening up new applications and markets, future trends need to be considered as well (“second,” “third,” and “fourth” generation materials, cf. EU Commission, 2012). This task is also addressed at, e.g., the recently started FP7 project FutureNanoNeeds attempting to deliver an overview on recent trends for next generation materials that are not on the market yet (www.futurenanoneeds.eu/).

However, to derive a prioritization on potential “relevant” ENMs and their release pathways to be addressed in ES1205, additional information is needed on production and application volumes.

PRODUCTION-VOLUME AND TRANSFER PATHWAYS INTO THE URBAN WATER SYSTEM

As a result of the lack of exposure data to receiving compartments, an assessment of release of ENMs into the environment by modeling the mass flow is currently the method of choice (e.g., Blaser et al., 2008; Mueller and Nowack, 2008; Petersen et al., 2011). Recent studies compared several peer reviewed publications and addressed the mass flow of ENMs into different compartments on a global level (e.g., Gottschalk et al., 2013; Keller et al., 2013). Most authors pronounce that the major source of modeling error is related to the uncertainty of production and application. Key input quantities, beside those mentioned in the previous section, are information from industry independent institutions, market surveys and interviews on production volumes, patents, and publications all related to limited insight to real market products. Hence, major challenges are associated with production and application information that are more representative than the current data available. Nevertheless, all of the authors cited in this paragraph seem to agree that the studies help to receive “bandwidth” information on expected environmental concentrations. This approach might be significantly improved, if the models are fed with more reliable input data.

But, how realistic is an improvement of the reliability of data on production volumes and applications? There are several factors that may interfere with the release of more detailed information, e.g., (1) an insufficient trust-based relationship between “public founded science” and “industry,” (2) market competition interests of individual producers, (3) worries about an “over critical” public perception of these numbers or in contrast to this (4) a gap between industrial “nano” promises and socioeconomic innovation payback from “day-to-day” applications, which becomes visible by detailed numbers.

Independently from the aforementioned reasons, this bottleneck can be considered as one of the most significant open

issue when the environmental relevance or the potential impact (defined here as occurrence in the environment combined with *in situ* toxicity) of ENMs is discussed. In addition, challenges on definitions and standardization further complicate this situation.

TRANSFORMATION AND RETENTION PROCESSES DURING SEWAGE PASSAGE/TREATMENT

Taking the results from Ag-NP spiking experiments in a combined sewer system (Kaegi et al., 2013) as an example, a very efficient transport of ENMs along the sewer channel, with a significant transformation of the particles, can be expected. Electron microscopic investigation revealed that Ag-NP were very quickly attached to the sewer biomass and transported as “complex colloids” along the sewer line underlining the importance of heteroaggregation, especially at high ionic strength and suspended solid content in wastewater. Thus, waste water transport and treatment facilities are major hubs controlling the release of ENMs to the environment. However, also in cities with a state of the art sewer infrastructure a fraction of the ENMs will be directly discharged to surface waters or to treatment facilities (e.g., constructed wetlands) through the stormwater overflow during heavy rain events.

The removal of different materials, including Ag-NP (Kaegi et al., 2011; Doolette et al., 2013; Li et al., 2013), ZnO (Ma et al., 2013), TiO₂ (Westerhoff et al., 2011), CeO₂ (Limbach et al., 2008) in wastewater treatment plants (WWTPs, mostly activated sludge processes) have been studied in detail, and consistently very high removal efficiencies of approximately 95% or more have been reported. Therefore, ENMs are very efficiently removed from the wastewater stream and accumulated in the sludge biomass during the wastewater treatment. Consequently, at the moment, low Ag-NP concentrations in the effluent are reported [order of magnitude: ng/L (Li et al., 2013)] and released to the surface water.

The agricultural application of sewage sludge as fertilizer may result in a release of the ENMs accumulated in the sludge to the soil and to surface water as well as to the groundwater. Whether increased loads of ENMs from the sewage sludge have any negative effect on soil properties, or whether the ENM from sewage sludge are increasingly taken up by plants is not fully understood, yet.

As an example, in urban wastewater systems the transformation of Ag-NP to silver sulfide (Ag₂S) is of prime interest due to dramatic reduction of the toxicity of the Ag-NP already after partial sulfidation (Reinsch et al., 2012). The sulfidation process already starts in the sewer system (Kaegi et al., 2013) which is readily explained by considerable concentration of bisulfide reported from sewer systems (Nielsen et al., 2008). Near complete sulfidation has been observed in the WWTP (activated sludge process) and also in digested sludge the speciation of Ag is strongly dominated by Ag₂S (Doolette et al., 2013; Lombi et al., 2013; Ma et al., 2013). These findings are consistent with the observation of nanoscale Ag₂S in sewage sludge collected from full scale WWTP (Kim et al., 2010). Ag₂S is very resistant toward oxidation with O₂ and only minor fraction of Ag⁺ have been released from Ag₂S over periods of several months

(Levard et al., 2011; Lombi et al., 2013). To follow these changes in speciation/fractionation of ENMs in wastewater, sludge, soils and sediments sophisticated analytical techniques have to be improved.

ANALYTICAL TECHNIQUE EVALUATION: CHALLENGES AND PERFORMANCES

The EC recommendation for a definition of ENMs (EU Commission, 2011) asks for number-based particle size distributions to classify a material as nano or not. However, this material classification strategy should not be applied equally to all sectors, e.g., environmental monitoring of NPs, where other properties than particle-number concentrations reveal importance.

In general an analytical strategy must be adapted to the analyte, the required information and a given matrix (von der Kammer et al., 2012) but in terms of ENMs in (urban) water systems a variety of analytical challenges need to be tackled: (1) to-be-expected low mass concentrations, (2) the presence of natural and incidental particles of similar size, shape, and composition, (3) dissolution, agglomeration, association (e.g., natural colloids, cells, proteins) and transformation of ENMs, as well as (4) a lack of certified reference materials (CRMs) allowing for method validation.

Regarding inorganic ENMs with a relatively high natural background concentration of certain elements (e.g., SiO₂, TiO₂, ZnO) or an interfering proportion of similar incidental particles (e.g., Pt and Pd from automotive catalysts), the differentiation from natural background or incidental particles is challenging. It might be achieved by, e.g., elemental composition and/or isotopic-ratio determination (Weber et al., 2009; Tuoriniemi et al., 2012), using specific impurities in the inorganic ENMs or in the particles of the natural background [e.g., La in natural CeO₂ (von der Kammer et al., 2012)] or would only be possible on a single-particle based analysis using electron microscopy or single particle inductively coupled plasma-mass spectrometry (Pace et al., 2012; Tuoriniemi et al., 2012). Referring to ENMs with vanishing particulate background concentrations [e.g., Au, Ag, although they might be formed naturally under certain conditions (Weber et al., 2009)], a direct measurement of particulate concentrations might be sufficient in cases when interference from natural sources are unlikely.

Next to analytical technical challenges, CRMs are mostly still missing—comprehensive method validation becomes difficult and alternative strategies need to be developed in their absence. The parts of an analytical method being most error-prone are sampling and sample preparation. Standard-operating procedures (SOPs) addressing the sample preparation and analysis for most relevant ENMs are missing.

Although the number of available analytical methods is increasing, method development is still challenging, especially in terms of environmental samples and toxicity data verification. Furthermore, method validation and result assessment is made difficult due to missing SOPs and CRMs. In our opinion cost-efficient and straightforward applicable analytical methods are needed providing valid results to evaluate whether ENMs are present in a given sample and at which concentration. A crucial prerequisite to reach this goal are “nano” CRMs

rectified in size and fraction-related quantity which are still lacking.

ECOTOXICITY-BASED CONSIDERATIONS

In recent years a large amount of time and effort as well as funding opportunities have focused on the investigation into the fate and effects of ENMs on human health and the environment. Although this research is novel and bespoke it is not necessarily the most useful when considering regulations and risk assessment (RA). There is also a lack of comparative studies with bulk products in order to identify possible nano-specific effects and assess the need for nano-specific regulations. Therefore, a lot of data available in the literature may not be applicable in the decision-making process on the need for nano-specific regulatory requirements or recommendations. In general, there is a consensus view that existing regulations should suffice.

The RA paradigm (hazard identification and characterization followed by exposure assessment and risk characterization) is considered appropriate (EFSA Scientific Committee, 2011). However, there may be a need for more specific guidance on certain aspects of their assessment. Toxicity often cannot be related to the actual size, the mass or the surface area of the single NPs or the NP agglomerates in exposure media even under standardized laboratory conditions. Therefore, it can be proposed that the toxicity is greatly influenced by other inherent and poorly understood properties of the particles that are not considered in standard guidance documents. While for *in vitro* studies the necessity to study interactions of ENMs and proteins but also other molecules in the exposure medium is of crucial importance in order to understand observed effects (Lynch et al., 2013) this is not yet commonly considered in studies using aquatic invertebrates. Important aspects that will need careful consideration in the future are the interactions of ENMs within the environmental compartments and combined effects with legacy chemicals (e.g., Farkas et al., 2012), correct dosimetry, formulation, and exposure route to organisms, characterization, and transformation in the environment (e.g., Ag-NPs: Impellitteri et al., 2013; Lombi et al., 2013) but also the possibility of food chain effects (e.g., Shu et al., 2010). All of which are difficult to analyse and quantify, therefore (along with the challenges regarding input quantities and analyses) making a realistic RA nearly impossible at the present time.

There is a need for nano predicted effect/no effect concentrations (PEC/PNECs) for receiving environmental compartments which form an essential part of RA (EU Commission, 2003; ECHA, 2008). Simple models to derive PECs and to address likely sinks need to be developed to compensate for the uncertainties in the data available on aspects of ENMs such as usage, concentrations (in products) and release into the environment and thereby support the generated ecotoxicity data leading to better and more realistic RA of this class of contaminants.

Despite a growing opinion that existing measures are suitable for the hazard and risk characterization of ENMs, nano risk tends to be treated on a case-by-case basis and this will not be sustainable in the future as more and more novel materials and products

come on the market. The scientific community is discussing but not yet ready to answer how to deal with new biological mechanisms and processes arising from novel nanostructures, eco-identity, Trojan-horse effect, etc. and how to make clear cut decisions on regulations.

CRITICAL APPRAISAL

To better coordinate and to structure scientific endeavors on ENM RA the following points should be considered in future evaluations:

- (1) For all ENMs studied so far, a removal efficiency >95% in WWTPs was shown. As a consequence sewage sludge pathways should be addressed.
- (2) In urban wastewater ENMs undergo different transformations reactions which generally reduce the reactivity. Hence, the properties and fate of transformed ENMs with regard to speciation and fractionation need to be addressed.
- (3) Studies on remobilization/speciation of ENMs from/in ashes, sediments, landfills, and soils are mostly lacking, this gap has to be addressed within the next years.
- (4) There is a challenge to differentiate between good ecotoxicity studies with, e.g., sufficient analytical verification and a high informative value and those that lack quality as well as environmental relevance. Most of the studies are effect and not mechanism focused. Studies that allow statements on *in situ* effects are lacking. These scientific drawbacks can be addressed by defining, e.g., basic quality criteria for studies with ENM.
- (5) Analytical methods tailored to selectively detect ENMs are important to study the interaction of ENMs understand mechanisms and processes (e.g., cell wall/membrane transfer of ENMs). Furthermore, “nano” CRMs certified in size and fraction related quantities are highly needed for method validation. For monitoring initiatives and ecotoxicity studies, the analytical methods including sample preparation protocols need to be easily applicable to complex matrices and operationally defined extraction methods [e.g., enrichment via cloud point extraction combined with atomic absorption spectroscopy (Hartmann and Schuster, 2013)], may help to allow for distinction between dissolved species and particles, if verified by other methods (Fabricius et al., 2014). As an example for Ag-NPs at the moment, total Ag content analyses seem appropriate for surface and groundwater monitoring. In future activities it will be very important to evaluate which ecotoxic effects can be expected if the total amount of Ag < 0.45 µm equals 100, 50, or 10% ENM and how the degree of transformation of the ENMs modulates the expected ecotoxicity. This may raise the need to derive nano specific PNECs and to compare these with existing PNECs of the receiving water bodies.
- (6) The access to data on production volumes and applications is poor. This is a major missing link for the prioritization of ENMs in environmental RAs and can only be counteracted by an improved trust-based relation between industries and scientists or a labeling of “nano” products, which show the potential to release a “relevant” nano fraction. However, this

is a discussion connected to many different challenges and driven by many different interests.

Although surface waters represent one of the most important receiving compartment for ENMs, based on the available scientific knowledge the authors representing the COST Action ES1205 ENTER do not see, at the moment, a general need for establishing nano-specific monitoring programs for surface waters. However, surface waters in the vicinity of production facilities may require increased attention.

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Potential for increased photosynthetic performance and crop productivity in response to climate change: role of CBFs and gibberellic acid

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We propose that targeting the enhanced photosynthetic performance associated with the cold acclimation of winter cultivars of rye (*Secale cereale* L.), wheat (*Triticum aestivum* L.), and *Brassica napus* L. may provide a novel approach to improve crop productivity under abiotic as well as biotic stress conditions. In support of this hypothesis, we provide the physiological, biochemical, and molecular evidence that the dwarf phenotype induced by cold acclimation is coupled to significant enhancement in photosynthetic performance, resistance to photoinhibition, and a decreased dependence on photoprotection through non-photochemical quenching which result in enhanced biomass production and ultimately increased seed yield. These system-wide changes at the levels of phenotype, physiology, and biochemistry appear to be governed by the family of C-repeat/dehydration-responsive family of transcription factors (CBF/DREB1). We relate this phenomenon to the semi-dwarf, gibberellic acid insensitive (GAI), cereal varieties developed during the “green revolution” of the early 1960s and 1970s. We suggest that genetic manipulation of the family of C-repeat/dehydration-responsive element binding transcription factors (CBF/DREB1) may provide a novel approach for the maintenance and perhaps even the enhancement of plant productivity under conditions of sub-optimal growth conditions predicted for our future climate.

Keywords: phenotypic plasticity, photosynthetic performance, crop productivity, CBFs, gibberellic acid, climate change

INTRODUCTION

The increase in the yield of major food crops since the mid-1950s has been achieved mainly through genetic improvement and increased use of agricultural inputs such as fertilizers, pesticides, and water (Murchie et al., 2009). Zhu et al. (2010) have suggested that the yield of major food crops since the last decade is increasing slowly, which may indicate that yield increase due to improved agricultural practices has reached an upper theoretical limit. Thus, it appears that the further enhancement in crop yield can only be achieved by enhancing genetic yield potential, that is, the seed yield that a crop can achieve per unit ground area under optimum growth conditions without biotic and abiotic stresses. The maximum potential biomass and grain yield that a plant can produce is determined essentially by the following five yield variables: (a) the amount of incident solar radiation available over the growing season of a plant, (b) the light interception efficiency, that is, the efficiency of the photosynthetic pigments to intercept photosynthetic active radiation, (c) the energy conversion efficiency, that is, the ratio of the biomass energy produced over a given period to the radiative energy intercepted by the canopy over the same period, (d) the translocation of photosynthates to sinks, as determined by sink strength, and (e) the partitioning

efficiency, that is, the amount of total biomass energy partitioned into seed production per unit ground area, also known as harvest index (HI) (Loomis and Amthor, 1999; Long et al., 2006; Zhu et al., 2010).

Since energy partitioning efficiency and light interception efficiency have approached the theoretical upper limit (Zhu et al., 2010), further increase in yield potential can only be achieved by an increase in the energy conversion efficiency into biomass. Since plant dry matter consists of about 40% carbon by weight, an increase in total biomass production can be achieved through enhanced photosynthetic carbon assimilation (Murchie et al., 2009). Although photosynthesis is the ultimate basis for the conversion of light energy into biomass and seed yield to date, improving photosynthetic carbon assimilation has played only a minor role in enhancing energy conversion to biomass and seed yield (Long et al., 2006; Zhu et al., 2010).

This is, in part, due to the important photoprotective mechanisms that have evolved in all photoautotrophs to protect the photosynthetic apparatus from irradiance that is in excess of that which can be utilized for either reductive CO₂ assimilation as well as reductive N and S assimilation (Adams III and Demmig-Adams, 1993; Demmig-Adams and Adams III, 1996;

Horton et al., 1996; Demmig-Adams et al., 1999; Niyogi, 1999; Horton, 2000, 2012; Horton and Ruban, 2005). Excess light is not only necessarily due to an increase in absolute actinic irradiance but is also generated with no change in absolute irradiance when coupled with either low temperature (Krause, 1994; Hüner et al., 1998; Ensminger et al., 2006; Farage et al., 2006; Takahashi and Murata, 2008; Hüner and Grodzinski, 2011) or other abiotic and biotic environmental stresses (Murata et al., 2007; Takahashi and Murata, 2008) by decreasing the photochemical efficiency of PSII. Consequently, the induction of photoprotective mechanisms to dissipate excess absorbed energy represents an important mechanism to balance the flux of energy absorbed and transformed into reducing power with the flux of energy utilized through the consumption of photosynthetically generated electrons by C, N, and S metabolism (Anderson et al., 1995; Hüner et al., 1998, 2013; Öquist and Hüner, 2003; Eberhard et al., 2008; Murchie et al., 2009; Hüner et al., 2012). Although the induction of photoprotective mechanisms reduces the efficiency of CO₂ assimilation and decreases biomass production, these photoprotective mechanisms are essential for plant survival against myriad environmental stresses (Kulheim et al., 2002; Sane et al., 2012). Even though inhibition of these photoprotective mechanisms would theoretically increase energy conversion efficiency in the very short-term, this is simply not an option for long-term survival of plants exposed to environmental conditions that fluctuate on an hourly, daily, and annual basis. Photoprotection from excess irradiance is essential for maximizing plant fitness and survival (Kulheim et al., 2002).

The increase in yield potential of major crops over the past 50 years occurred, by and large, as a consequence of improved partitioning efficiency and light interception efficiency of crop plants (Long et al., 2006; Murchie et al., 2009; Zhu et al., 2010). Increased partitioning efficiency has been accomplished through the release of semi-dwarf cultivars producing higher numbers of seeds per plant and an increase in HI. The new wheat varieties associated with the “green revolution” and introduced in the 20th century were generally shorter and exhibited an increased grain yield at the expense of straw biomass, that is, exhibited enhanced HI. The *gai* mutant alleles that control this shorter phenotype and increased HI encode mutant forms of the gibberellin response transcription factor, GAI (Peng et al., 1999). Increased light interception efficiency is a consequence of increased leaf area index associated with the development of the semi-dwarf cultivars with improved lodging resistance against the adverse weather conditions, such as rain, wind, and hail (Peng et al., 1999; Long et al., 2006; Murchie et al., 2009). Recently, it was reported that the rice cytokinin GATA transcription factor1 encoded by *Cga1* governs a dwarf phenotype as well as chloroplast development in rice (Hudson et al., 2013). Thus, altered *Cga1* expression appears to mimic the effects of altered gene expression in GA signaling associated with the elite “green revolution” wheat varieties.

Presently, we summarize experimental evidence that targeting the dwarf phenotype, and enhanced photosynthetic performance typically associated with the cold acclimated (CA) state of winter cereals and *Brassica napus* may represent a novel approach to improve crop yield and productivity. We show that the requirement for cold acclimation to enhance photosynthetic

performance can be circumvented by overexpression of the C-repeat/dehydration responsive family of transcription factors. We suggest that this approach may provide important insights into potential molecular approaches focused on at least the maintenance or, perhaps, even the enhancement of plant productivity under sub-optimal growth conditions predicted to be associated with future climate change.

COLD ACCLIMATION INCREASES PHOTOSYNTHETIC PERFORMANCE

Previous studies have reported that CA winter cultivars of rye (Figure 1A), wheat, barley, Brassica, spinach, and *Arabidopsis thaliana* are characterized by an increased photosynthetic capacity relative to non-acclimated (NA) controls. Furthermore, the temperature response curves for both CO₂ assimilation (Figure 1B) and photosynthetic electron transport (ETR) (Figure 1C) indicate that CA plants exhibit higher rates at all temperatures between 5 and 25°C relative to NA controls (Figures 1B,C) (Hüner et al., 1998; Öquist and Hüner, 2003; Dahal et al., 2012a,b). This has been accounted for by the up-regulation of carbon metabolism as a consequence of increased gene expression and activities of CO₂-fixing enzyme, Rubisco (Hurry and Hüner, 1991; Hurry et al., 1994, 1995, 2000; Hüner et al., 1998; Strand et al., 1999; Öquist and Hüner, 2003; Dahal et al., 2012a,b) as well as enhanced activities of the cytosolic, sucrose biosynthetic enzymes, cFBPase, and SPS (Hurry et al., 1995a; Strand et al., 1999; Savitch et al., 2000a; Rapacz et al., 2008; Dahal et al., 2012a,b) in response to low growth temperature. In addition, winter cultivars of wheat (Savitch et al., 2000a; Leonardos et al., 2003) and *Arabidopsis thaliana* (Stitt and Hurry, 2002) combine an enhanced sink capacity with increased rates of sucrose export in response to cold acclimation. The major portion of this sucrose is stored as fructans in the crown tissue and leaf mesophyll cell vacuoles during cold acclimation of winter cereals (Pollock and Cairns, 1991; Savitch et al., 2000a). Consequently, cold acclimation of winter wheat, winter rape (Hurry et al., 1995a), and *Arabidopsis thaliana* (Stitt and Hurry, 2002) results in enhanced P_i cycling and increased capacity for RuBP regeneration. Concomitantly, cold acclimation of winter cereals suppresses photorespiration (Savitch et al., 2000b) and stimulates carbon export rates from source leaves (Leonardos et al., 2003). Consequently, the process of cold acclimation of winter cereals appears to co-ordinate system-wide readjustments in plant metabolism in feed-forward stimulation of photosynthetic CO₂ fixation due to enhanced source-sink activities and increased capacity for carbon translocation (Hurry et al., 1995a; Strand et al., 1999; Stitt and Hurry, 2002; Leonardos et al., 2003). This is supported by a detailed, comparative metabolomics study of CA vs. NA *Arabidopsis thaliana* (Gray and Heath, 2005).

These adjustments at the physiological, biochemical, and molecular levels in response to cold acclimation are associated with coordinated changes in leaf anatomy and plant phenotype (Hüner, 1985; Gray et al., 1996, 1997; Strand et al., 1999; Dahal et al., 2012a,b) and induction of freezing tolerance in cold-tolerant species (Sarhan et al., 1997; Pocock et al., 2001; Thomashow, 2001; Savitch et al., 2005; Rapacz et al., 2008; Theocharis et al., 2012). With respect to phenotypic plasticity,

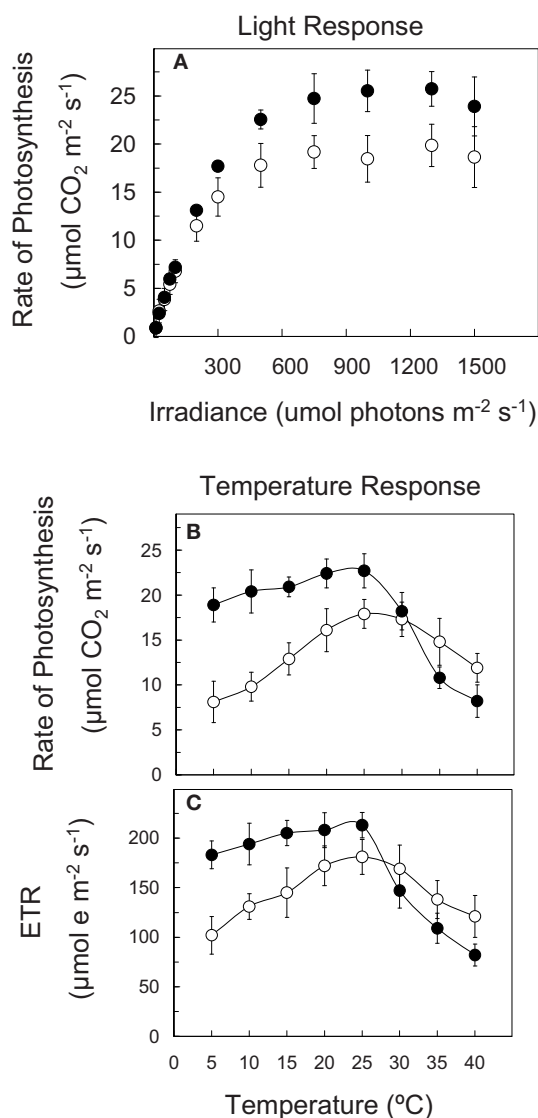
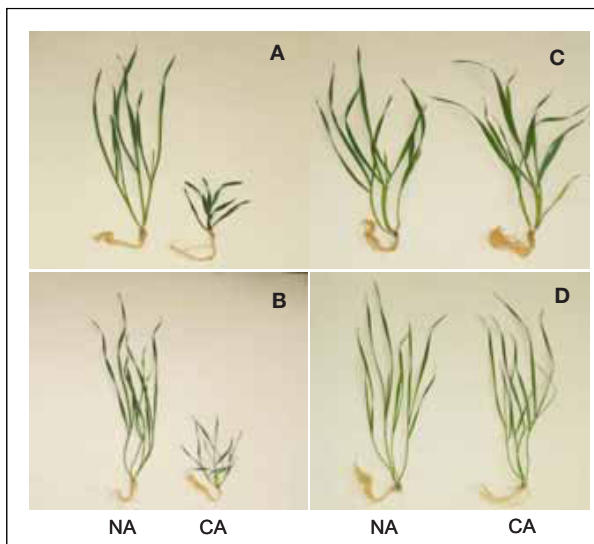


FIGURE 1 | Light and temperature response curves for winter rye (*Secale cereale* L. cv Musketeer). NA Musketeer (open symbols) was grown at 20°C and an irradiance of $250 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ whereas CA Musketeer (closed symbols) was grown at 5°C and $250 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 16 h/8 h light/dark photoperiod. **(A)** Light response curves for CO_2 assimilation were measured at the growth temperature under ambient CO_2 conditions. Temperature response curves for CO_2 assimilation **(B)** and ETR **(C)** represent light saturated rates under ambient CO_2 conditions. All data are from Dahal et al. (2012a,b,c) with permission.

cold acclimation of winter rye, winter wheat, and *Brassica napus* (Figure 2) as well as spinach and *Arabidopsis thaliana* generally results in a compact, dwarf phenotype (Hüner, 1985; Boese and Hüner, 1990; Strand et al., 1999; Savitch et al., 2005; Gorsuch et al., 2010a,b; Dahal et al., 2012a,b).

Previously, it has been assumed that the induction of the dwarf phenotype in overwintering herbaceous plants is regulated by low temperature (Levitt, 1980). However, we have shown that this is not the case. In fact, the phenotypic plasticity associated with cold acclimation of winter cultivars is regulated by the redox state of

Rye and Wheat Phenotypes



Brassica Phenotypes

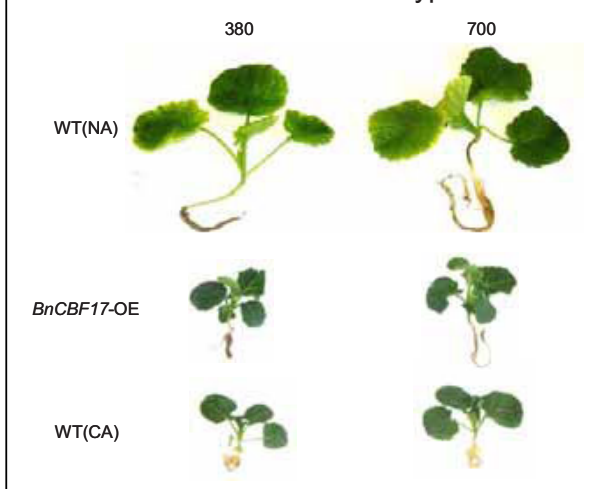


FIGURE 2 | The effect of cold acclimation on plant phenotype. All plants were photographed at similar physiological states based on comparative growth kinetics. See Dahal et al. (2012a,b,c), for details. **(A)** NA and CA winter rye (*Secale cereale* L. cv Musketeer). **(B)** NA and CA Norstar winter wheat (*Triticum aestivum* L. cv Norstar). **(C)** NA and CA spring rye (*Secale cereale* L. cv SR4A). **(D)** NA and CA spring wheat (*Triticum aestivum* L. cv Katepwa). NA *Brassica napus* L. cv Westar WT and the *BnCBF17* overexpressor (*BnCBF17-OE*) were grown at 20°C and an irradiance of $250 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 16 h/8 h light/dark photoperiod at either ambient (380 ppm CO_2) or elevated CO_2 (700 ppm CO_2). CA *Brassica napus* L. cv Westar WT was grown at 5°C and an irradiance of $250 \mu\text{mol photons m}^{-2} \text{ s}^{-1}$ with a 16 h/8 h light/dark photoperiod at either ambient (380 ppm CO_2) or elevated CO_2 (700 ppm CO_2). Photographs are taken from Dahal et al. (2012a,b) with permission.

the chloroplast measured as excitation pressure (Gray et al., 1997; Hüner et al., 1998, 2012, 2013; Wilson et al., 2006; Kurepin et al., 2013). In addition, *WCS19*, a nuclear encoded gene originally associated with wheat freezing tolerance, has also been reported

to be regulated by excitation pressure rather than by low temperature (Gray et al., 1997; NDong et al., 2001). Excitation pressure, estimated *in vivo* using Chl a fluorescence induction (Dietz et al., 1985; Hüner et al., 1998), is a quantitative measure of the proportion of closed PSII reaction centers due to an imbalance between energy absorbed vs. energy either utilized through metabolism and growth or dissipated as heat. Excitation pressure represents a chloroplast redox signal which emanates from the photosynthetic ETR chain and regulates not only the organization and composition of the photosynthetic apparatus but also phenotypic plasticity (Anderson et al., 1995; Hüner et al., 1998, 2003, 2012, 2013; Enslinger et al., 2006; Rosso et al., 2009; Kurepin et al., 2013).

The CA dwarf phenotype is also associated with altered leaf mesophyll cell ultrastructure and increased leaf thickness. The former is characterized by an increase in cytoplasmic volume combined with a decrease in vacuolar volume as estimated from cross sectional areas of transmission electron micrographs which are correlated with increased specific leaf weight relative to NA winter rye and *Arabidopsis thaliana* (Hüner et al., 1984; Strand et al., 1999). The latter can be accounted for by either increases in leaf mesophyll cell size (Hüner, 1985; Gorsuch et al., 2010a,b) or increases in palisade mesophyll layers (Boese and Hüner, 1990, 1992; Dahal et al., 2012a). These changes in leaf morphology are accompanied by an increase in the leaf protein content (Table 1) as well as an increase in the contents of sucrose and other structural carbohydrates (Guy et al., 1992; Hurry et al., 1995b; Strand et al., 1999, 2003; Savitch et al., 2000a,b; Gorsuch et al., 2010b). Consequently, the absolute shoot biomasses of the CA dwarf plants are comparable to the shoot biomasses of the NA plants with the larger, extended phenotype (Table 1). This indicates a significant increase in total energy per unit plant volume during the autumn cold acclimation period which is critical for winter survival and enhanced seed yield the following spring (Dahal et al., 2012b). Furthermore, the increased photosynthetic capacity exhibited by CA plants is, in part, due to the

increased photosynthetic apparatus per unit leaf area (Dahal et al., 2012b). Functionally, this leads to an increased capacity to utilize absorbed light energy which keeps a greater proportion of PSII reaction centers open, that is, lowers excitation pressure (Hüner et al., 2003). This, in turn, results in a lower probability for light-dependent inhibition of photosynthesis due to photoinhibition (Krause, 1994, 1988; Osmond, 1994; Melis, 1999; Murata et al., 2007) under suboptimal growth conditions (Hurry and Hüner, 1992; Hüner et al., 1993; Gray et al., 1996, 1997). Although the co-ordination of such system-wide adjustments in photosynthesis, carbon metabolism, translocation, and phenotype appear to be correlated with the modulation of chloroplast excitation pressure, the precise molecular mechanism(s) that govern such complex, system-wide changes have yet to be elucidated. This remains a major challenge for future research.

However, the ability to co-ordinate system-wide adjustments in photosynthetic performance in response to low growth temperature appears to be cultivar dependent. In contrast to winter cereals, spring cultivars do not exhibit this change in phenotype but rather maintain an elongated phenotype upon cold acclimation (Figure 2) with minimal changes in SLW and leaf protein content (Table 1) even though spring varieties are able to grow at low temperature (Dahal et al., 2012b, 2013b). Most likely this is due to the differential vernalization requirements for winter vs. spring varieties (Sung and Amasino, 2005; Oliver et al., 2009; Ko et al., 2010; Trevaskis, 2010). However, CA spring cereals exhibit a significant inhibition in photosynthetic capacity coupled with a much higher susceptibility to photoinhibition compared to CA winter cereals (Hurry and Hüner, 1991; Pocock et al., 2001; Dahal et al., 2012b).

COLD ACCLIMATION MINIMIZES DEPENDENCE ON NPQ FOR PHOTOPROTECTION

It is estimated that under optimal growth conditions only about 4.6% of the initial energy that impinges the leaf surface is conserved as fixed carbon and plant biomass (Zhu et al., 2010;

Table 1 | Effects of cold acclimation on growth and photosynthesis.

Growth characteristic	Acclimation state	Musketeer	SR4A	Brassica WT	BnCBF17
Growth rate (day ⁻¹)	NA	0.225 ± 0.002	0.233 ± 0.003	nd	nd
	CA	0.074 ± 0.002	0.078 ± 0.002	nd	nd
<i>A_{sat}</i>	NA	18.0 ± 2.8	22.2 ± 2.3	15.0 ± 3.1	23.1 ± 2.0
	CA	27.1 ± 2.5	15.1 ± 1.8	22.5 ± 2.5	nd
SLW (g m ⁻²)	NA	37 ± 6	33 ± 5	27 ± 3	50 ± 6
	CA	90 ± 11	40 ± 4	63 ± 5	nd
Shoot dry mass (mg/plant)	NA	279 ± 12	337 ± 22	103 ± 6	122 ± 10
	CA	264 ± 32	349 ± 42	117 ± 12	nd
Leaf Protein (g m ⁻²)	NA	3.67 ± 0.32	3.92 ± 0.44	3.65 ± 0.33	16.30 ± 1.50
	CA	10.6 ± 1.13	3.53 ± 0.33	17.5 ± 1.40	nd
WUE (A/g _s)	NA	34 ± 5	29 ± 5	48 ± 6	63 ± 4
	CA	94 ± 12	32 ± 4	87 ± 13	nd

Winter rye (*Secale cereale* L. cv Musketeer); Spring rye (*Secale cereale* L. cv SR4A); WT, wild type; Brassica napus L. cv Westar; BnCBF17, CBF overexpressor of Brassica napus; SLW, specific leaf weight; *A_{sat}*, light saturated rate of CO₂ assimilation (μmol CO₂ m⁻² s⁻¹); WUE, water use efficiency calculated as the ratio of light-saturated rates of CO₂ assimilation (A) and stomatal conductance (g_s). All data are from Dahal et al. (2012b, 2013b).

Dahal et al., 2013a). A major challenge in photosynthesis research remains the enhancement in the conversion of absorbed light energy into plant biomass (Murchie et al., 2009; Zhu et al., 2010). Much recent research addressing this issue has focused primarily on targeted genetic approaches either to alter the structure and composition of the photosynthetic photosystems and their associated antenna complexes, to alter the structure of the CO₂ fixing enzyme, Rubisco, in order to reduce the rates of photorespiration in C₃ crop plants (Spreitzer and Salvucci, 2002; Long et al., 2006; Zhu et al., 2010) or to increase the potential for C₄ photosynthesis in C₃ plants such as rice (Edwards et al., 2008). In contrast to these targeted approaches, we propose that exploitation of the system-wide adjustments in photosynthetic performance orchestrated by cold acclimation provides a novel approach to enhance plant biomass production by co-ordinating source-sink demand which concomitantly minimizes energy loss through NPQ. To assess relative dependence on NPQ, one may compare the balance between the efficiency for light utilization for CO₂ assimilation with the efficiency for energy dissipation through NPQ (Dahal et al., 2012a). The former can be measured as the apparent number of photons required to close 50% of PSII reaction centers (Figure 3), and the latter as the apparent number of photons required to induce one unit of NPQ (Dahal et al., 2012a). An increased capacity for the utilization of photosynthetic reductants to assimilate CO₂, will be reflected in an increase in the quantum requirement, that is, a decrease in the initial slope of the light response curve for excitation pressure. For example, Figure 4C (open symbols) illustrates that CA *Brassica napus* WT exhibits a lower initial slope than that observed for NA *Brassica napus* WT (Figure 4A, open symbols). This indicates that cold acclimation increases the number photons required to close a PSII reaction centers by 30–50% compared to NA *Brassica*. Concomitantly, this is associated with a 30–50% increase in the number of photons required to induce photoprotection through NPQ in CA (Figure 4F) vs. NA *Brassica napus* WT (Figure 4D). Thus, CA *Brassica napus* exhibits a lower dependence on NPQ for photoprotection than NA *Brassica napus* due to increased rates of photosynthetic ETR and photosynthetic CO₂ assimilation (Dahal et al., 2012a). Similar results have been reported for CA vs. NA winter rye and winter wheat which translates into a 60% increase in seed yield per plant (Dahal et al., 2013b). Thus, cold acclimation of winter rye, winter wheat, and *Brassica napus* establishes a new homeostatic state which is characterized by an increased photosynthetic capacity for CO₂ assimilation and its conversion into biomass or energy per unit plant volume and seed production in wheat under suboptimal growth conditions (Dahal et al., 2013a,b).

In addition to enhanced photosynthetic performance and superior resistance to photoinhibition (Powles, 1984; Long et al., 1994; Edelman and Mattoo, 2008), cold acclimation suppresses stomatal conductance and transpiration rates by 30–40% regardless of the measuring temperature. The suppression of stomatal conductance observed upon cold acclimation is due, at least in part, to a decrease in leaf stomatal density. (Dahal et al., 2012a,b, 2013b). Consequently, CA plants also exhibit approximately a 3-fold increase in leaf water use efficiency (WUE) relative to NA controls (Dahal et al., 2012a,b, 2013b). However, the increase in

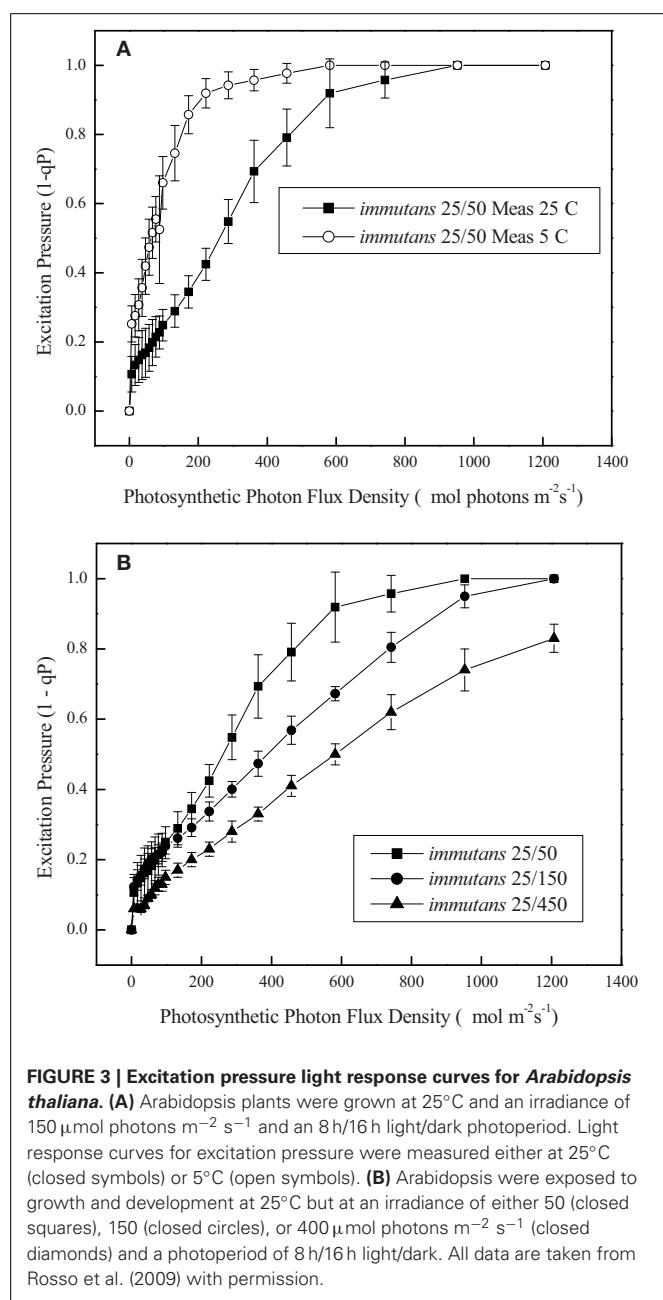
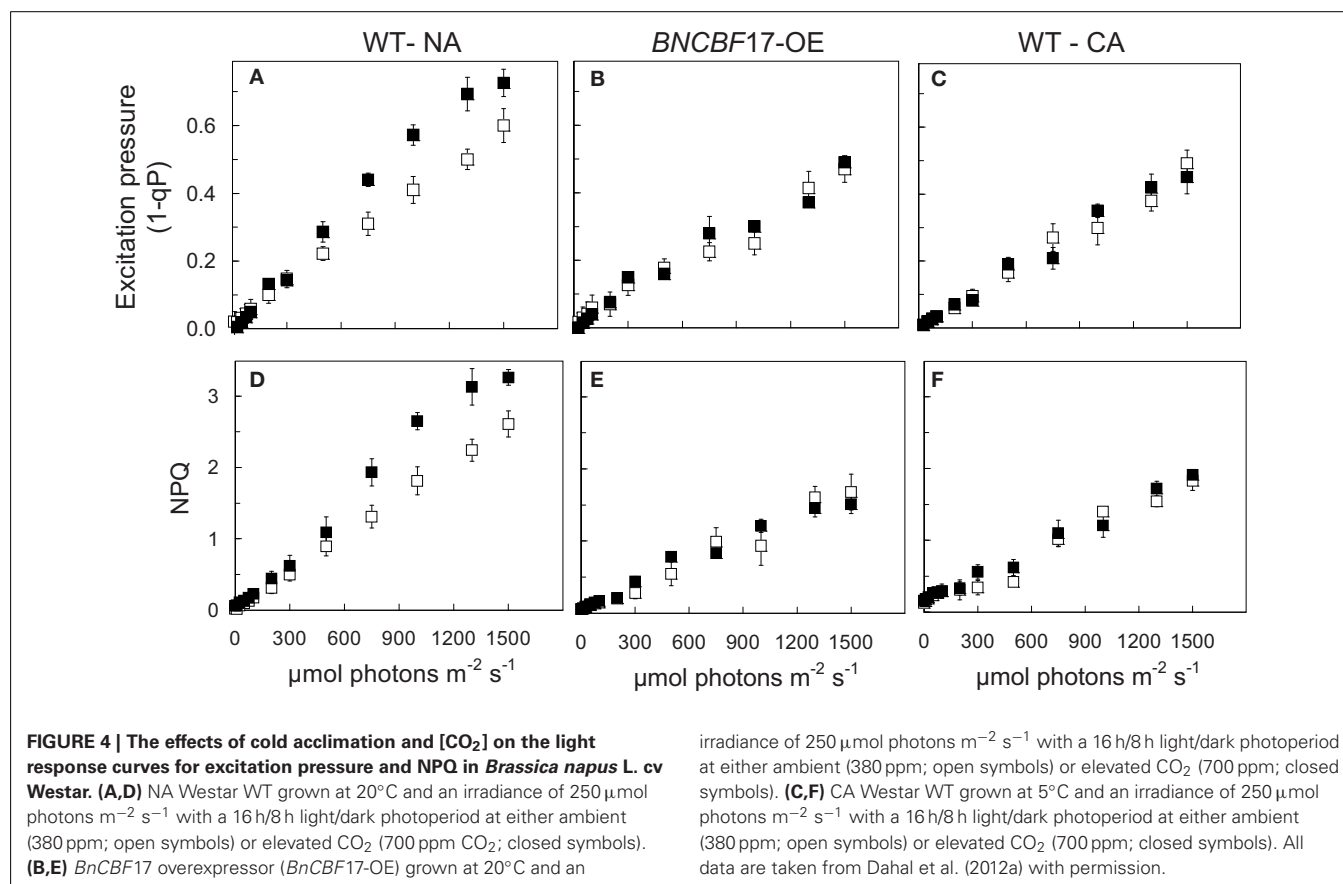


FIGURE 3 | Excitation pressure light response curves for *Arabidopsis thaliana*. (A) *Arabidopsis* plants were grown at 25°C and an irradiance of 150 μmol photons m⁻² s⁻¹ and an 8 h/16 h light/dark photoperiod. Light response curves for excitation pressure were measured either at 25°C (closed symbols) or 5°C (open symbols). (B) *Arabidopsis* were exposed to growth and development at 25°C but at an irradiance of either 50 (closed squares), 150 (closed circles), or 400 μmol photons m⁻² s⁻¹ (closed diamonds) and a photoperiod of 8 h/16 h light/dark. All data are taken from Rosso et al. (2009) with permission.

WUE appears primarily due to a combination of a decrease in stomatal density combined with the observed increase in light saturated rates of photosynthesis.

ENHANCED PHOTOSYNTHETIC PERFORMANCE IS MAINTAINED DURING LONG-TERM GROWTH AT ELEVATED CO₂

It has been established that a short-term shift of C₃ species from ambient (380 μmol C mol⁻¹) to elevated CO₂ (700 μmol C mol⁻¹) results in an increase in the rates of CO₂ assimilation (Long et al., 2004; Ainsworth and Rogers, 2007; Dahal et al., 2012c). This stimulation of photosynthesis in C₃ plants due to elevated CO₂ occurs because Rubisco is CO₂ substrate-limited at



ambient CO₂ (Long et al., 2004; Tcherkez et al., 2006) and photorespiration is suppressed since CO₂ is a competitive inhibitor of the oxygenation of RuBP by Rubisco (Long et al., 2004).

In contrast to a short-term shift, long-term growth and development of C3 plants at high CO₂ may lead to end product inhibition of photosynthesis due to the accumulation of sucrose in the cytosol (Stitt and Quick, 1989; Foyer et al., 1990). This feedback inhibition of photosynthetic capacity in response to growth and development at elevated CO₂ levels may result from the combination of chloroplast Pi-limitations and the down regulation of the expression and activities of key regulatory photosynthetic enzymes (Sharkey and Vanderveer, 1989; Stitt and Quick, 1989; Drake et al., 1997; Long et al., 2004). The results in **Figure 5A** illustrate the inhibition light saturated rates of CO₂ assimilation in WT *Brassica napus* grown at 25°C at either ambient (open symbols) or elevated CO₂ (closed symbols). Consequently, WT *Brassica napus* grown at elevated CO₂ exhibits higher excitation pressure under CO₂ saturation conditions (**Figure 5B**, closed symbols) than WT *Brassica napus* grown at ambient CO₂ conditions (**Figure 5B**, open symbols). However, CA *Brassica napus* WT does not suffer from feedback limited photosynthesis as a consequence of growth and development at elevated CO₂ (Dahal et al., 2012a).

Similar to CA *Brassica napus* WT, CA winter cereals exhibit a 30–40% increase in light and CO₂-saturated rates of photosynthesis at both ambient and elevated CO₂. This was accompanied by a 35–50% decrease in excitation pressure and

non-photochemical energy dissipation. Concomitantly, biomass increased by 28–46% and grain yield per plant by 60% (Dahal et al., 2013b). Thus, CA *Brassica napus*, winter rye, and wheat are able to maintain their superior photosynthetic performance with respect to light and CO₂ saturated rates of CO₂ assimilation relative to NA controls and do not exhibit feedback inhibition of photosynthesis when exposed to growth and development at elevated CO₂. However, since this is not the case for spring rye and spring wheat cultivars, the potential for enhancement of photosynthetic performance and grain yield of cereals during long-term growth at elevated CO₂ appears to be cultivar dependent (Dahal et al., 2013b).

Several reports have shown that a previous exposure to one type of abiotic stress can lead to enhanced tolerance to other abiotic and biotic stresses. Cold acclimation of overwintering cereals results in increased systemic resistance to plant infection by psychrophilic fungi (Hiilovaara-Teijo et al., 1999; Griffith and Yaish, 2004). Changes in temperature and CO₂ levels affect the interactions between plant and insect pollinators as well as plant and insect herbivores due to alterations in plant phenology (DeLucia et al., 2012). Kane et al. (2013) compared the global gene expression of NA and CA Norstar winter wheat grown at either ambient or elevated CO₂ conditions. Global gene expression analyses using microarrays combined with bioinformatics indicated that gene expression in general was more sensitive to elevated CO₂ in NA, with 1022 genes in total, in contrast to 372 genes differentially regulated by CO₂ in CA Norstar (Kane et al.,

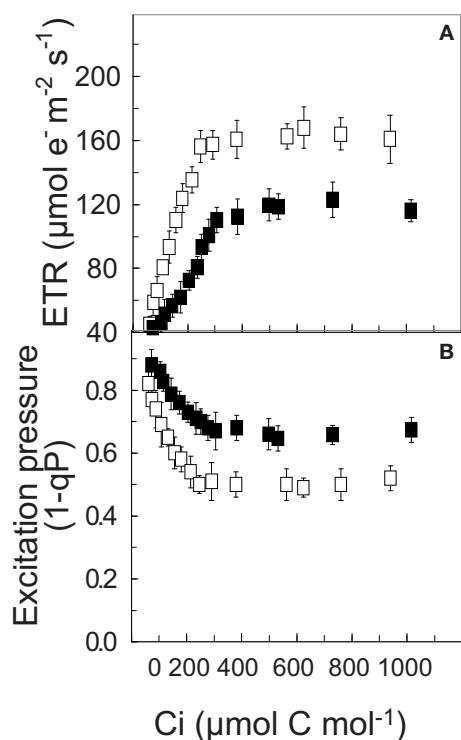


FIGURE 5 | The effects long-term growth [CO₂] on the CO₂ response curves for ETR (A) and excitation pressure (B) for WT *Brassica napus* L. cv Westar. Plants were grown at 20°C and an irradiance of 250 μmol photons m⁻² s⁻¹ with a 16 h/8 h light/dark photoperiod at either ambient (380 ppm; open symbols) or elevated CO₂ (700 ppm CO₂; closed symbols). All data are taken from Dahal et al. (2012a) with permission.

2013). However, the most striking effect of long-term growth at high CO₂ was the fact that 121 of the 768 genes down-regulated by growth at elevated CO₂ in NA Norstar are either pathogen-related or associated with systemic acquired resistance which demonstrates a major negative impact on pathogen and biotic stress defense mechanisms in NA winter wheat upon growth at high CO₂. In contrast, CA Norstar exhibits minimal down regulation of the same genes associated with pathogen resistance and biotic stress defense. In fact, growth of CA Norstar at elevated CO₂ caused an induction of genes involved in the biosynthesis of phytoalexins and anthocyanins as well as an up-regulation of chalcone synthase and dihydroflavonol-4-reductase (Kane et al., 2013). In addition, genes involved in photoprotection (ELIPs) and the maintenance of chloroplast stability (chaperonins) were significantly up-regulated during growth and development of CA Norstar at elevated CO₂ (Kane et al., 2013). This is consistent with the physiological data which indicate that cold acclimation generally enhances photoprotection and the stability of the photosynthetic apparatus even under elevated CO₂ conditions. Thus, based on the global microarray and bioinformatics analyses (Kane et al., 2013), we predict that cold acclimation not only enhances photosynthetic performance, it should also enhance inherent resistance to biotic stress. However, the latter must be confirmed experimentally and is most likely cultivar dependent.

OVEREXPRESSION OF CBFs CIRCUMVENTS THE REQUIREMENT FOR COLD ACCLIMATION

The expression of CBFs (C-repeat binding factors) initiate the expression of *COR* genes necessary to acquire freezing tolerance and induce a dwarf phenotype that is comparable to CA plants (Figure 2) (Jaglo-Ottosen et al., 1998; Medina et al., 1999; Zarka et al., 2003; Benedict et al., 2006; Chinnusamy et al., 2007; Thomashow, 2010; Lee and Thomashow, 2012). However, in addition to mimicking the freezing tolerance response, growth of CBF overexpressors at 25°C also mimicks the enhanced photosynthetic capacity (Figures 4B,E), increased plant biomass and WUE (Table 1) typically associated with cold acclimation. This is consistent with the stimulation of the rates of photosynthetic ETR and CO₂ assimilation reported for the *BnCBF17* overexpressor for *Brassica napus* (Dahal et al., 2012a). Furthermore, similar to CA *Brassica napus* WT, the *BnCBF17* overexpressor also does not exhibit feedback inhibition of photosynthesis during long-term growth at elevated CO₂ (Dahal et al., 2012a). Thus, both cold acclimation and *BnCBF17* overexpression overcome the feedback-limited photosynthesis observed in NA control WT *Brassica* plants. Thus, we conclude that overexpression of *BnCBF17* circumvents the requirement for cold acclimation to co-ordinate the system-wide adjustments in plant phenotype, photosynthetic performance and source-sink relationships in *Brassica napus*. Although CBF overexpression clearly alters the physiology of the shoot, little or no information is available with respect to the impact of CBF overexpression on root morphology, physiology, and nutrient uptake.

CA winter rye not only exhibits significantly higher rates of CO₂ assimilation (Figure 1B, closed symbols) and ETR (Figure 1C, closed symbols) but also a lower temperature sensitivity at all temperatures between 5 and 25°C than NA winter rye (Figure 1, open symbols). Similar trends were observed for NA and CA *Brassica napus* WT (Dahal et al., 2012b). However, most surprising is the fact that the temperature response curves for CO₂ assimilation and photosynthetic ETR for CA *Brassica napus* are mimicked by simply overexpressing *BnCBF17* even when these plants are grown at 25°C (Dahal et al., 2012b). To our knowledge, this represents the first report of such a novel manipulation of the temperature sensitivity of photosynthesis.

However, in contrast to overexpression of *BnCBF17*, overexpression of *BnCBF5* resulted in minimal changes in photosynthetic performance *Brassica napus* (Savitch et al., 2005). Clearly, the enhanced photosynthetic performance induced by CBF overexpression appears to be dependent upon the specific member of this family of transcription factors that is overexpressed. Thus, a more detailed assessment of the effects of overexpression of each member of the CBF family of transcription factors is required.

THE POTENTIAL ROLE OF CBFs

A major focus of research which attempts to elucidate the molecular mechanisms underlying plant cold acclimation and freezing tolerance has been on changes in cell membrane structure as reflected in changes in membrane lipid and fatty acid content and composition. An inherent assumption is that the plant cell membrane is the primary site that determines the potential of plants to acclimate to low temperature and consequently

exhibit maximum freezing tolerance (Levitt, 1980; Steponkus, 1984; Guy, 1990; Murata and Los, 1997; Thomashow, 2001; Los and Murata, 2002; Chinnusamy et al., 2007). In cyanobacteria, two-component histidine kinases activated by changes in membrane viscosity have been shown to stimulate the expression of specific fatty acid desaturases. This results in the modulation of membrane fluidity in response to low temperature stress in cyanobacteria (Los and Murata, 2002; Xin, 2002; Los et al., 2013). Low temperature-induced alterations in the physical structure of plant cell membranes also activates Ca^{2+} channels and rapidly generates a Ca^{2+} signal (Monroy et al., 1998; Plieth et al., 1999; Orvar et al., 2000). The rapid increase in cytosolic Ca^{2+} activates a cytosolic protein kinase whose substrate is ICE1. The phosphorylation of ICE1 is required for the induction of a family CBF transcription factors which regulate the expression of COR genes essential for acquisition of maximal freezing tolerance (Jaglo-Ottosen et al., 1998; Medina et al., 1999; Zarka et al., 2003; Benedict et al., 2006; Chinnusamy et al., 2007; Thomashow, 2010; Lee and Thomashow, 2012). The expression of *CBF3* in *Arabidopsis thaliana* is positively regulated by the constitutively expressed ICE1 gene, the product of which binds to multiple regulatory elements present in the *CBF3* promoter and stimulates its transcription (Chinnusamy et al., 2007; Thomashow, 2010). The action of ICE1, in turn, is tightly regulated by SIZ1, a SUMO E3 ligase, and HOS1 which is a RING finger E3 ligase (Lee et al., 2001; Zhu et al., 2004; Dong et al., 2006; Miura et al., 2007; Hua, 2009; Miura and Ohta, 2010; Thomashow, 2010).

Enhanced freezing tolerance has been reported in plants such as *A. thaliana*, canola (*Brassica napus* L.), tomato (*Solanum lycopersicum* L.), and poplar (*Populus balsamifera* subsp. *trichocarpa*) in which CBFs have been over-expressed (Jaglo-Ottosen et al., 1998; Hsieh et al., 2002; Savitch et al., 2005; Benedict et al., 2006). In herbaceous plants, CBF overexpression is typically associated with the dwarf phenotype observed during cold acclimation even though the plant has been grown at warm temperatures (Hsieh et al., 2002; Savitch et al., 2005; Achard et al., 2008; Huang et al., 2009; Dahal et al., 2013a). Thus, the CBF regulon not only regulates freezing tolerance but also has dramatic effects on plant development that are considered to be controlled by the family of plant phytochromes as well as phytohormones (Franklin, 2009; Kurepin et al., 2013).

All photoautotrophic organisms sense changes in light quality as a “biogenic signal” through photoreceptors such as the phytochromes and cryptochromes to regulate photomorphogenesis (Pogson et al., 2008; Sakamoto et al., 2008; Waters and Langdale, 2009; Jarvis and Lopez-Juez, 2013). However, photoautotrophs also sense changes in light intensity as fluctuations in energy input for photosynthesis as an “operational signal” to maintain a cellular energy balance that is, photostasis (Hüner et al., 2003, 2012; Pogson et al., 2008). Photoautotrophs must balance the extremely fast rates of cellular energy input through the temperature-insensitive photochemical reactions of photosystem I and photosystem II with the slower, and temperature-dependent enzyme processes involved in either energy dissipation as heat or energy utilization through primary C, N, and S assimilation (Foyer et al., 2000; Foyer and Noctor, 2002; Murchie et al., 2009; Hüner and Grodzinski, 2011; Hüner et al., 2012, 2013). The

regulation of the phytochrome-dependent, photomorphogenic signal transduction pathways (Franklin and Whitelam, 2007; Pogson et al., 2008; Franklin, 2009) as well as the retrograde sensing/signaling pathways between the chloroplast and the nucleus (Koussevitzky et al., 2000; Nott et al., 2006; Lim et al., 2007; Fernandez and Strand, 2008; Pogson et al., 2008; Woodson and Chory, 2008) involved in remodeling of the photosynthetic apparatus are both light- and temperature-dependent (Li et al., 1993; Franklin, 2009; Kim and Apel, 2013; Hüner et al., 2012, 2013). Thus, cross-talk between the biogenic signaling pathways and the complex operational signaling networks must exist since both govern plant plasticity in response to an ever-changing environment. Elucidation of nature of this complex integration of light sensing/signaling networks remains a major challenge for future research.

Recently, we proposed that CBFs act as master regulators of cold acclimation and photosynthetic performance which integrate both the upstream and downstream signals (Kurepin et al., 2013). In our model illustrated in **Figure 6**, we propose that redox input signals from chloroplasts (green), manifested as modulation of excitation pressure, are transduced to the nucleus (red) via retrograde regulation and stimulate CBF expression. Recently, we reported that *CBF3* in *Arabidopsis thaliana* is regulated by excitation pressure rather than low temperature *per se* (Bode, 2012; Kurepin et al., 2013). Furthermore, we suggest that the phenotype and physiological properties of CA plants occurs by the stimulation of CBF gene expression to induce not only freezing tolerance but also genes associated with photosynthesis, cytosolic carbon metabolism and respiration in addition to the activation of GA2ox genes which decreases the levels of growth-active GAs. Growth-active GAs normally bind to DELLA proteins to de-repress genes involved in cell growth and stem elongation (Peng and Harberd, 1997; Peng et al., 1997, 1999). Thus, accumulation of growth-inactive GAs maintains levels of DELLA proteins such growth and stem elongation are repressed which generates a dwarf phenotype. This dwarf phenotype exhibits enhanced photosynthetic performance and increased dry biomass per unit plant volume (Savitch et al., 2005; Dahal et al., 2012a, 2013a,b) coupled with enhanced seed yield in CA wheat (Dahal et al., 2013b). We conclude that plants sense low temperature through a complex sensing/signaling network which integrates changes in temperature and actinic light intensity through modulation of operational signals emanating from the chloroplast due to chloroplast redox imbalance or excitation pressure (Hüner et al., 1998; Ensminger et al., 2006; Hüner et al., 2012, 2013; Kurepin et al., 2013). Thus, the chloroplast should not only be considered the primary energy in transformer but also a major cellular energy sensor for detecting changes in the environment (Hüner et al., 1998; Pfannschmidt, 2003; Murchie et al., 2009). However, operational chloroplast redox signaling must be integrated with regulation associated changes in light quality sensed through photoreceptors such as phytochrome (Franklin and Whitelam, 2007; Franklin, 2009) as well as through specific cell membrane, low temperature sensors (Murata and Los, 1997; Plieth et al., 1999; Knight and Knight, 2000; Los and Murata, 2002; Los et al., 2013) in order to establish a new CA, homeostatic state.

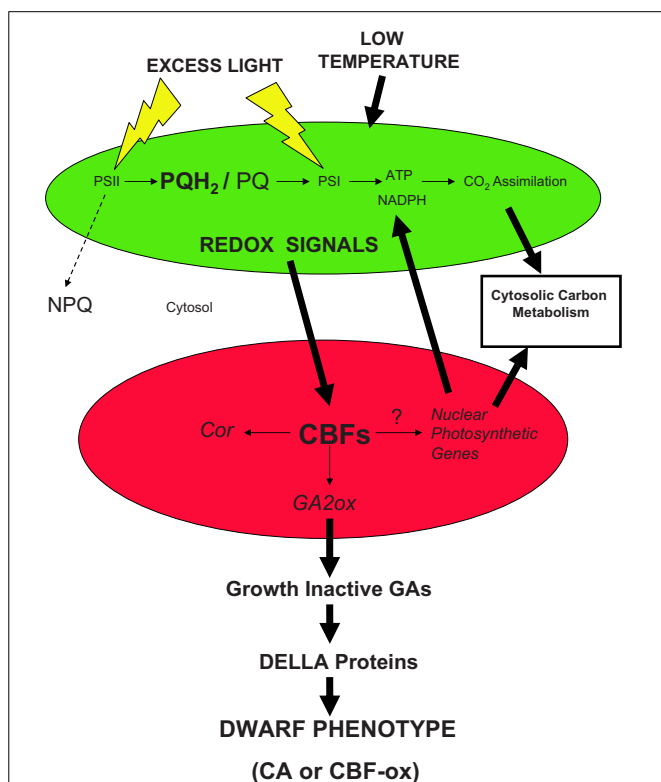


FIGURE 6 | A proposed model illustrating the role of CBFs in governing the system-wide integration of excitation pressure, photosynthetic capacity and the dwarf phenotype.

Growth of plants either under excess light or low temperature poises the intersystem photosynthetic electron transport chain of the chloroplast (green) in a reduced state as indicated by the accumulation of plastoquinol (PQH₂) relative to plastoquinone (PQ). This is due to an imbalance between the flux of energy absorbed through extremely fast, temperature-independent photochemistry occurring within the PSII and PSI reaction centers relative to the energy utilized either through much slower, temperature-sensitive biochemical reactions involved in CO₂ assimilation, cytosolic carbon metabolism, and export or the energy dissipated as heat through NPQ. Such an energy imbalance increases excitation pressure. Through retrograde signaling pathways, the myriad redox signals generated by the chloroplast due to the increased excitation pressure are transduced to the nucleus (red) to modulate *CBF* expression. This leads to the activation not only of *COR* genes important in freezing tolerance but also the activation of nuclear encoded photosynthetic genes associated with thylakoid membrane protein complexes involved in photosynthetic electron transport as well as enzymes of the Calvin–Benson Cycle and cytosolic sucrose biosynthesis. The latter results in the increased capacity for CO₂ assimilation upon growth under high excitation pressure. DELLA proteins are repressors of stem elongation. Growth-active GAs stimulate stem elongation by activating the breakdown of DELLA proteins, and thus, de-repressing stem elongation. *CBFs* affect the biosynthetic pathways for GA biosynthesis by activating nuclear encoded *GA2ox* genes which predisposes plants to accumulate growth-inactive GAs (Kurepin et al., 2013). Consequently, activation of *CBFs* by excitation pressure results in the accumulation of growth inactive GAs which maintains the plant in a repressed growth state due to the accumulation of DELLA proteins. As illustrated in the model, the low growth temperature or high light requirement usually needed to elicit enhanced photosynthetic capacity and the dwarf phenotype can be circumvented by overexpression over-expression of *CBFs* (*CBF-ox*). The enhanced photosynthetic capacity induced either by excitation pressure or overexpression of *CBFs*, minimizes the requirement for photoprotection of the chloroplast through NPQ as illustrated by the broken arrow. The mechanism by which *CBFs* activate the expression of known nuclear photosynthetic genes is presently unknown.

CBFs, CLIMATE CHANGE, AND CROP PRODUCTIVITY

The intergovernmental panel on climate change has predicted that the atmospheric CO₂ concentration will double from present 380 μmol C mol⁻¹ to ca. 700 μmol C mol⁻¹ by the end of the twenty-first century which may be coupled to an increase in average global temperature (IPCC, 2007). The predicted increase in atmospheric CO₂ and drastic temperature changes associated with global climate change may increase the severity of water stress as well as the incidence of biotic stresses (Hatfield et al., 2011; DeLucia et al., 2012; Vandegeer et al., 2012). Such sub-optimal growth conditions due to climate change may substantially affect the photosynthetic performance of plants and hence, plant biomass production and crop seed yield. To add to the complexity of the effects of climate change on crop productivity, the sub-optimal growth conditions associated with climate change is occurring at a time when the world population is likely to increase by about 30% by 2050 (UN Report, 2011). It has been predicted that the world food demand will double in the next 20 years due to increased world population as well as increased per capita food consumption (Murchie et al., 2009). This has triggered an immediate need to substantially enhance seed yield and productivity of major food crops such as rice, wheat, and maize to meet the nutritional demand of the increasing population. This urgent need is exacerbated by the fact that the projected increased food demand is occurring at a time when continued losses of prime agricultural land is occurring due to urbanization, desertification in most of the developing countries, an increasing proportion of grain is being diverted for animal feed and biofuel generation which are combined with the suboptimal growth conditions for crop production due to climate change (Murchie et al., 2009; Zhu et al., 2010).

During the 1960s and 1970s wheat yields worldwide increased significantly due to the development of new varieties and increased availability and use of N fertilizers. This led to the “green revolution” which was characterized by short, sturdy, semi-dwarf genotypes that exhibit an increased HI and a decreased tendency to lodge (Peng et al., 1999). Subsequent molecular and genetic analyses of these semi-dwarf varieties showed that this phenotype was conferred by dominant mutant dwarfing alleles which are orthologs of the *Gibberellic Acid Insensitive* gene (*GAI*) of *Arabidopsis thaliana* (Peng et al., 1999). In wild type, *GAI* represses stem elongation at low endogenous GA levels but under high endogenous GA levels, GA binds to *GAI* and de-represses the inhibitory effect of *GAI* which results in normal stem elongation and an elongated phenotype (Peng et al., 1997, 1999; Peng and Harberd, 1997). In the dwarf plants, the mutant gene, *gai*, is present which does not bind endogenous GA. Consequently, mutants retain a dwarf phenotype irrespective of endogenous GA levels.

Since growth active GAs stimulate stem elongation (Hopkins and Hüner, 2008), application of exogenous GAs to *CBF*-overexpressing plants rescues the dwarf phenotype. In contrast, application of other plant hormones known to affect shoot growth does not rescue the dwarf phenotype (Hsieh et al., 2002; Achard et al., 2008). Published reports indicate that overexpression of *CBFs* results in increased levels of DELLA proteins (Achard et al., 2008; Kurepin et al., 2013) which repress stem

elongation and leads to a dwarf phenotype (Peng and Harberd, 1997; Peng et al., 1997). Recent evidence indicates that excitation pressure not only regulates the expression of photosynthetic genes (Hüner et al., 1998; Rosso et al., 2009) but also activates *AtCBF3* expression in *Arabidopsis thaliana* (Bode, 2012; Kurepin et al., 2013). Since growth-active GAs stimulate the degradation of DELLA proteins (Achard et al., 2008), the accumulation of DELLA proteins, in part, reflect the increased expression of *GA2ox* genes which leads to the accumulation of growth-inactive GAs (Kurepin et al., 2013). Thus, the published evidence is consistent with the notion that excitation pressure governs the dwarf phenotype through the activation *CBF* gene expression which, in turn, may control the expression of *GA2ox* genes which consequently reduces the accumulation of growth active GAs in CA plants as well as plants grown under excessive irradiance (Gray et al., 1997; Achard et al., 2008; Kurepin et al., 2013). Consequently, plants grown under either excessive irradiance or low temperature exhibit a dwarf phenotype which is mimicked by overexpression of *CBFs* (Gilmour et al., 2000; Savitch et al., 2005; Dahal et al., 2012a) (Figure 6).

The model illustrated in Figure 6 is consistent with the thesis that photosynthesis and the chloroplast have a dual role—not only do they represent the major energy transformers of sunlight into biomass, they also govern a broad range of physiological and plant developmental processes which have a direct impact on phenotypic plasticity. This model is consistent with the “grand design of photosynthesis” first proposed by Arnon (1982) and subsequently supported by a series of reviews in the last 20 years focused on photoacclimation (Anderson et al., 1995), acclimation to low temperature and light (Hüner et al., 1998; Pfannschmidt, 2003), acclimation and cell death (Mullineaux and Baker, 2010) as well as plant growth and productivity (Murchie et al., 2009; Kurepin et al., 2013).

The maintenance of crop yield stability through enhanced tolerance to environmental stresses such as drought, low and high temperature, as well as biotic stress associated with global climate change remains a crucial challenge to maximize future crop productivity worldwide (Powell et al., 2012). We suggest that targeting the *CBF* family of transcription factors in major crop species may be a novel approach to improve crop productivity through increased photosynthetic performance coupled with increased WUE and the potential for enhanced resistance to biotic stress. However, an important caveat to this approach is that low temperature induction of *CBFs* in *Arabidopsis thaliana* also activates the expression of Flowering Locus C (*FLC*), a negative regulator of flowering (Seo et al., 2009). This results in a significant delay in flowering time which reflects an evolutionary mechanism to prevent premature floral development during the late fall or early spring seasons. Similarly, overexpression of *AtCBF3* delayed the onset of bolting by 4 to 9 days at 20°C in *Arabidopsis* (Gilmour et al., 2000). Such a delay in flowering time has been confirmed in subsequent studies (Magome et al., 2004; Seo et al., 2009). Furthermore, seed yield also appears to be reduced in *CBF* overexpressors compared to wild type when grown at warm temperatures (Liu et al., 1998; Gilmour et al., 2000). Thus, when grown under optimal conditions, the increased photosynthetic performance induced by *CBF* overexpression may

not translate into increased seed yield. However, it is crucial to compare the conversion of plant biomass into seed yield in *CBF* overexpressors with wild type plants when grown under suboptimal growth conditions. The prediction is that the *CBF* overexpressor should out perform the wild type under such conditions. Further research is required to confirm this prediction.

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Compound-specific isotope analysis of diesel fuels in a forensic investigation

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Compound-specific isotope analysis (CSIA) offers great potential as a tool to provide chemical evidence in a forensic investigation. Many attempts to trace environmental oil spills were successful where isotopic values were particularly distinct. However, difficulties arise when a large data set is analyzed and the isotopic differences between samples are subtle. In the present study, discrimination of diesel oils involved in a diesel theft case was carried out to infer the relatedness of the samples to potential source samples. This discriminatory analysis used a suite of hydrocarbon diagnostic indices, alkanes, to generate carbon and hydrogen isotopic data of the compositions of the compounds which were then processed using multivariate statistical analyses to infer the relatedness of the data set. The results from this analysis were put into context by comparing the data with the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ of alkanes in commercial diesel samples obtained from various locations in the South Island of New Zealand. Based on the isotopic character of the alkanes, it is suggested that diesel fuels involved in the diesel theft case were distinguishable. This manuscript shows that CSIA when used in tandem with multivariate statistical analysis provide a defensible means to differentiate and source-apportion qualitatively similar oils at the molecular level. This approach was able to overcome confounding challenges posed by the near single-point source of origin, i.e., the very subtle differences in isotopic values between the samples.

Keywords: diesel fuel, alkane, compound-specific isotope analysis (CSIA), principal component analysis (PCA), hierarchical clustering analysis (HCA)

INTRODUCTION

Compound-specific isotopic analysis (CSIA) is a technique that is becoming increasingly popular as a forensic tool to measure stable isotope composition of chemical compounds, such as hydrocarbons, which can be likened to fingerprinting at the molecular level. From a forensic perspective, it is important to infer a link between a sample to a suspected source(s) by obtaining chemical information from both data sets or to differentiate the sample in question from the other samples of known origin, i.e., a population study where an informed assessment can be made by obtaining the profiles of the sample population to provide context for the data. In other words, stable isotope fingerprinting provides the resolution to the question of whether two compounds or substances are distinguishable, i.e., un-related. If the two samples are indistinguishable this supports the hypothesis that they are related. Although variations in stable isotope ratios are generally very small they are robust and have allowed workers in numerous fields to formulate links between samples (e.g., Brand and Coplen, 2012). With the help of stable isotope fingerprinting, forensic scientists are able to support inferences to link a person to an event, a crime scene, or a criminal organization, based on a unique characteristic of some physical evidence. Traceability of diesel fuel, i.e.,

to demonstrate any linkages, or relationships, between diesel samples is very important to ascertain culpability in a diesel theft case. Therefore, to have methods to unambiguously characterize, identify and assign sources is key to withstand the legal scrutiny in the court of law.

Current techniques for the characterization of oil products generally use gas chromatography (GC) to separate and quantify the different molecular species. Crude oils from different sources exhibit different characteristics in terms of the ratios of molecular species. Processing of the oil changes these characteristics, e.g., the high molecular weight pentacyclic terpenes and steranes are generally removed during the refining process whereas the diamondoids (adamantanes and diamantanes) are found in most petroleum products (Wang et al., 2006). Recent advances in the interfacing of GC to isotope ratio mass spectrometers offers the potential to enhance the fingerprinting capability of the GC techniques by harnessing the discriminating power offered through isotope ratio measurements (Turner et al., 2006). Petroleum-derived hydrocarbon samples of different origin and/or history have been shown to be distinguishable by CSIA based on the stable isotope signatures albeit the differences obtained are subtle (Philp et al., 2002; Smallwood et al., 2002).

Petroleum products are generally mixtures of volatile, semi-volatile and refractory compounds. These will exhibit quite different isotopic evolution during processing and/ degradation with some compounds changing very little and others exhibiting very significant change (e.g., Muhammad et al., 2015). Furthermore, once leaving the refinery or following release into the environment, the original isotopic signatures may change due to fractionation or mixing processes. This tremendous isotopic variability offers a strong forensic tool in comparing/contrasting different samples of the same compound (contaminant). Thus, careful selection of target molecules offers the means to identify the origin of the petroleum products.

A few studies have incorporated multivariate statistics to correlate and differentiate petroleum hydrocarbons to its source(s) using stable isotope fingerprints (Boyd and Coffin, 2004; Boyd et al., 2006). These statistical techniques are suitable to be applied in the area of environmental hydrocarbon fingerprinting due to the large number of samples and variables involved. Principal component analysis (PCA) is an exploratory statistical analysis which is frequently applied in this area due to its ability in detecting potential group tendency within a sample set; i.e., to assign a class membership to each sample. PCA can also reveal underlying features in the dataset that are responsible for the detected classification (Pasadakis et al., 2008). Another statistical

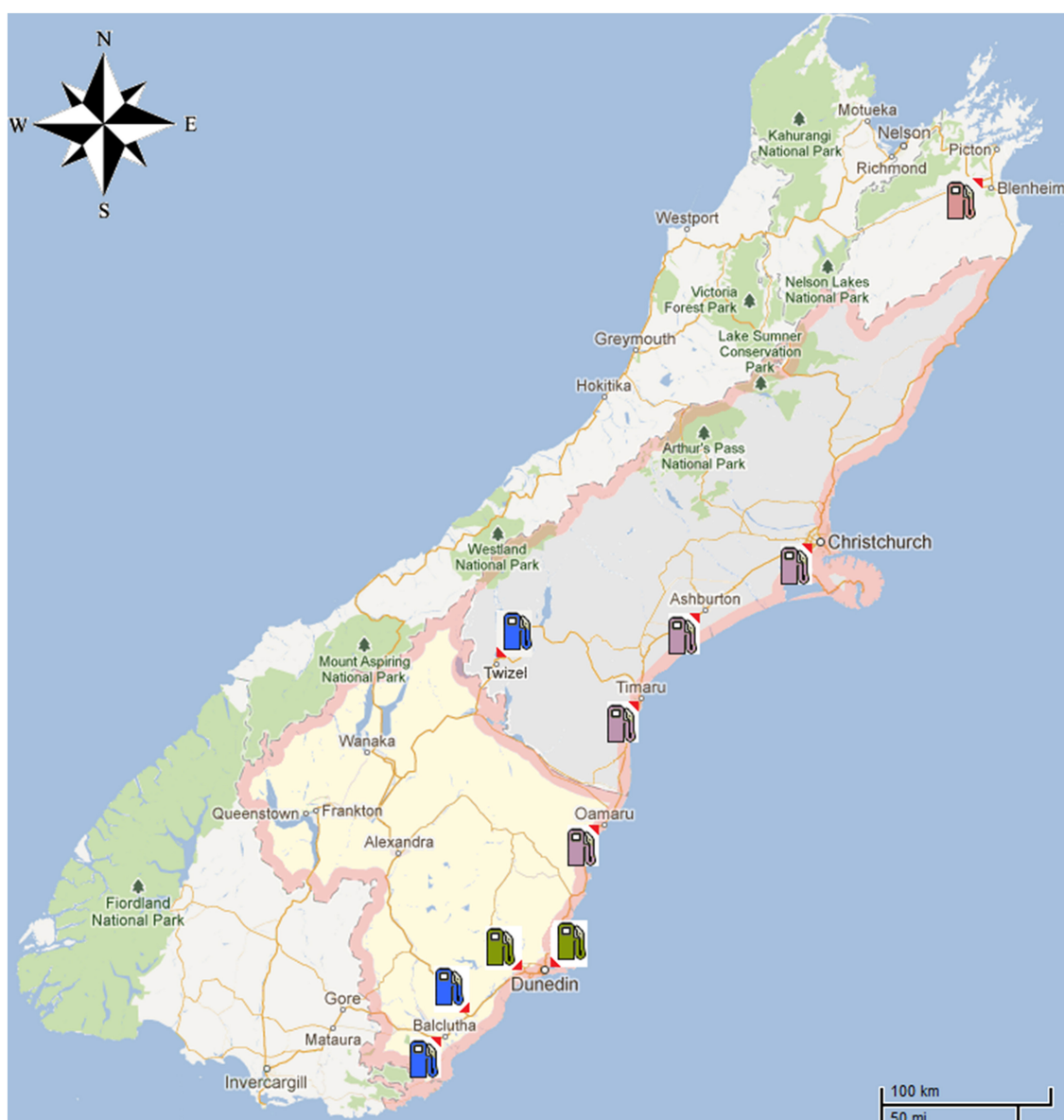


FIGURE 1 | A map of the South Island of New Zealand showing the diesel theft location as well as the area where the 45 diesel samples were obtained from commercial service

stations. Locations were marked as follows: Blenheim (red gas pump icon), Dunedin City (green gas pump icon), Canterbury region and North Otago (blue gas pump icon), South Otago and Twizel (blue gas pump icon).

technique which is also frequently utilized in oil-source correlation work is hierarchical clustering analysis (HCA). Models are built based on distance connectivity between samples in a multidimensional space spanned by the original variables in this type of analysis. The objective is to assign each sample to a group of objects in a stepwise manner where extensive hierarchies of clusters merge with each other at certain distances (Muhammad et al., 2013).

The objective of this study is to carry out forensic fingerprinting of diesel fuels based on the isotopic character of a suite of hydrocarbon compounds as diagnostic indices. The relationship between samples in the data set was then explored using multivariate statistical analyses to provide a defensible means of classification.

MATERIALS AND METHODS

SAMPLE DETAILS AND PREPARATION

Case samples were provided by the Blenheim Police Department and arrived in our laboratory in separate, labeled bottles with polypropylene screwed caps. The samples were labeled in two different categories: (1) Control (2 samples); (2) GKT ### (9 samples). The Control samples were taken from the storage tanks located at the ski field in Blenheim (**Figure 1**) where the diesel theft occurred and the GKT samples with different denominations were sub-sampled from containers found with the suspect. The diesel from the ski field was winterized diesel. This type of diesel is formulated to withstand the freezing temperature during the winter to avoid it coagulating and becoming solid in fuel lines. Fluidity was enhanced by adding a small amount of

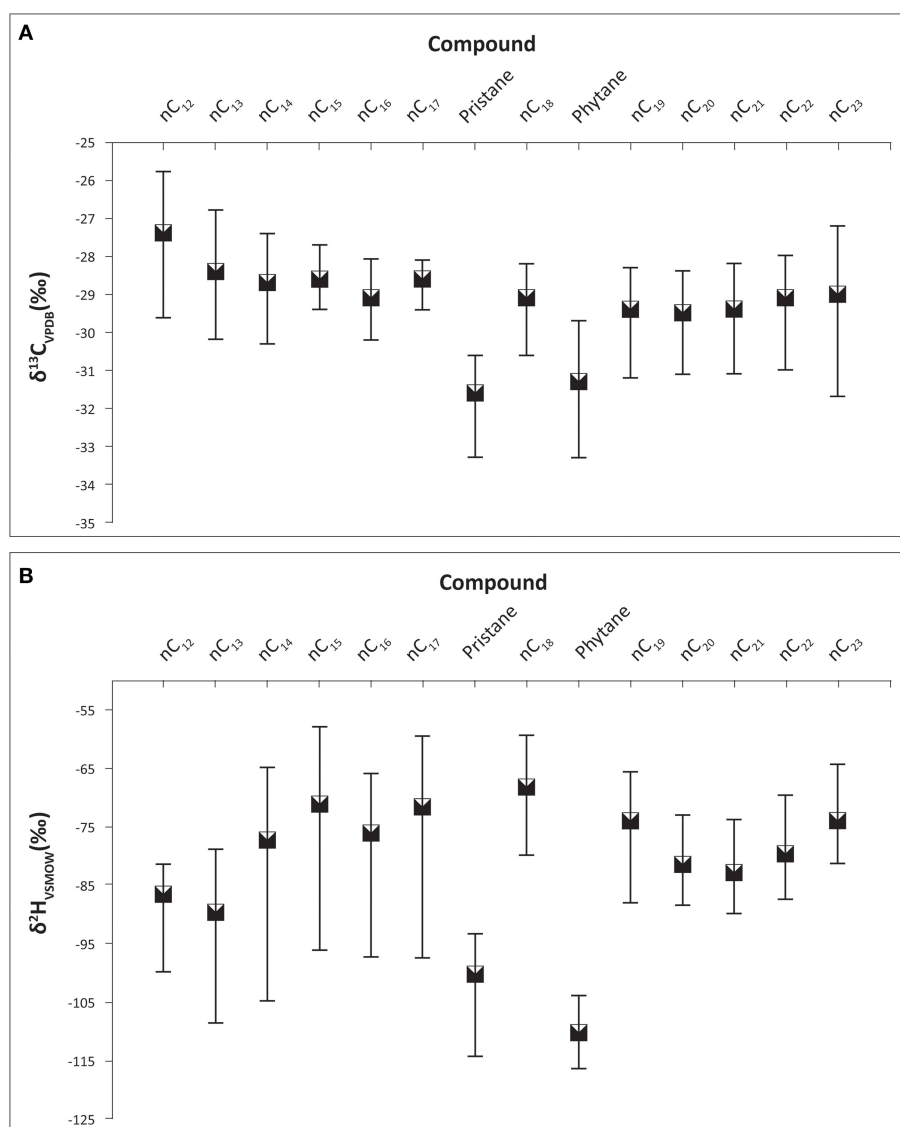


FIGURE 2 | Carbon and hydrogen isotope ratios of the alkane compounds found in Control, GKT, and South Island of New Zealand diesel samples. Bars represent the range of isotope values for each compound. **(A)** The range of carbon isotope compositions

of the alkanes within these diesel samples were between -33.3 to -25.8 ‰. **(B)** The range of hydrogen isotope compositions of the alkanes within these diesel samples were between -116.6 to -58.1 ‰.

additive (i.e., lower molecular weight hydrocarbons) to improve liquefaction. In this regard, these winterized diesels would possibly show distinct fingerprints from the regular diesels found at service stations. No information was provided as to the origin of the samples or, e.g., whether the samples were replicates as the sampling details were kept secret according to the provisions of law.

To provide context for the analysis of the diesel fuel involved in the theft case, a further 45 commercial diesel samples were obtained from different service stations located around the South Island of New Zealand (Figure 1). These samples came from areas such as Dunedin City, North and South Otago, Twizel, Christchurch City and South Canterbury (Muhammad et al., 2013).

The diesel fuels were prepared for GC analysis by sub-sampling 20 μ L of each sample and dispensed using a micro syringe into a GC vial which was then diluted to 2 mL with *n*-pentane. All samples were prepared in duplicate.

GC ANALYSIS

Gas chromatographic and isotope analyses were obtained on at least two aliquots of each sample and each analysis was an average of at least three measurements. Compound specific carbon and

hydrogen isotope ratios were determined using a Trace Ultra-gas chromatograph (Thermo, Milan, Italy) coupled to a Delta^{plus} XP isotope ratio mass spectrometer (Thermo, Bremen, Germany) via a high temperature conversion furnace, heated to 940°C and 1450°C for carbon and hydrogen analyses, respectively. The injection mode used was splitless with the temperature set at 300°C. Compounds were separated using a HP-1 GC column (30 m, 0.32 mm i.d., 0.25 μ m film thickness: J&W scientific). Identification of the analytes was made prior to isotope analysis using GC-FID by comparing the retention time of DRH-008S-R2 hydrocarbon standard solution (AccuStandard, Connecticut, USA), which contains 35 *n*-alkane compounds in chloroform, with that of the samples. The GC analytical conditions for both instruments were set to be the same throughout each run to avoid misrepresentation. The carrier gas for the analysis was helium with a constant flow of 1.5 ml/min. The parameters for the analytical run were as follows: initial oven temperature 50°C, initial

Table 1 | Statistics of the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ value of individual alkanes in Control, GKT, and South Island of New Zealand samples.

	N	Range	Minimum	Maximum	Mean	SD	Variance
<i>n</i> C ₁₂ ¹³ C	56	3.9	-29.6	-25.8	-27.6	0.9	0.8
<i>n</i> C ₁₃ ¹³ C	56	3.3	-30.2	-26.8	-28.4	0.9	0.8
<i>n</i> C ₁₄ ¹³ C	56	2.8	-30.3	-27.4	-28.6	0.6	0.3
<i>n</i> C ₁₅ ¹³ C	56	1.6	-29.4	-27.7	-28.6	0.4	0.1
<i>n</i> C ₁₆ ¹³ C	56	2.3	-30.2	-27.9	-29.1	0.5	0.2
<i>n</i> C ₁₇ ¹³ C	56	1.4	-29.5	-28.1	-28.6	0.4	0.1
Pris ¹³ C	56	4.1	-33.3	-29.2	-31.3	1.1	1.1
<i>n</i> C ₁₈ ¹³ C	56	2.4	-30.6	-28.2	-29.1	0.6	0.4
Phy ¹³ C	56	4.9	-33.3	-28.3	-30.9	1.2	1.6
<i>n</i> C ₁₉ ¹³ C	56	2.9	-31.2	-28.3	-29.3	0.7	0.5
<i>n</i> C ₂₀ ¹³ C	56	2.7	-31.1	-28.4	-29.4	0.6	0.4
<i>n</i> C ₂₁ ¹³ C	56	2.9	-31.1	-28.2	-29.4	0.8	0.6
<i>n</i> C ₂₂ ¹³ C	56	3.0	-31.0	-28.0	-29.2	0.8	0.6
<i>n</i> C ₂₃ ¹³ C	56	4.5	-31.7	-27.2	-29.1	1.2	1.3
<i>n</i> C ₁₂ ² H	56	54.6	-100.0	-45.5	-82.1	11.4	130.1
<i>n</i> C ₁₃ ² H	56	35.0	-108.9	-74.0	-88.4	7.9	62.7
<i>n</i> C ₁₄ ² H	56	39.9	-105.1	-65.1	-78.2	10.6	111.4
<i>n</i> C ₁₅ ² H	56	38.1	-96.2	-58.1	-72.8	11.3	126.7
<i>n</i> C ₁₆ ² H	56	31.3	-97.5	-66.2	-77.6	8.6	73.6
<i>n</i> C ₁₇ ² H	56	37.9	-97.6	-59.7	-73.3	11.0	122.0
Pris ² H	56	29.2	-114.6	-85.4	-100.6	5.9	35.0
<i>n</i> C ₁₈ ² H	56	22.4	-82.1	-59.7	-69.8	6.0	36.3
Phy ² H	56	39.1	-116.6	-77.5	-105.5	10.3	105.1
<i>n</i> C ₁₉ ² H	56	22.5	-88.3	-65.8	-74.3	4.8	23.2
<i>n</i> C ₂₀ ² H	56	19.8	-88.5	-68.7	-80.0	5.3	28.6
<i>n</i> C ₂₁ ² H	56	17.6	-90.0	-72.4	-82.3	4.8	22.8
<i>n</i> C ₂₂ ² H	56	19.1	-87.5	-68.5	-78.6	6.1	36.9
<i>n</i> C ₂₃ ² H	56	33.9	-86.4	-52.5	-73.7	6.8	46.4

Table 2 | Component matrix which shows variables and their contribution to the variance in the data set of Control and South Island of New Zealand samples.

	Component ^a				
	1	2	3	4	5
<i>n</i> C ₁₂ ¹³ C	0.728	0.445	0.153	-0.262	0.368
<i>n</i> C ₁₃ ¹³ C	0.651	0.494	0.211	-0.184	0.324
<i>n</i> C ₁₄ ¹³ C	0.713	0.454	-0.110	-0.253	0.304
<i>n</i> C ₁₅ ¹³ C	0.144	0.774	0.216	0.323	0.268
<i>n</i> C ₁₆ ¹³ C	0.264	0.678	0.302	0.478	0.176
<i>n</i> C ₁₇ ¹³ C	0.457	0.479	0.520	0.301	-0.294
Pris ¹³ C	0.884	0.173	-0.079	0.268	0.182
<i>n</i> C ₁₈ ¹³ C	0.827	0.429	-0.085	-0.103	-0.042
Phy ¹³ C	0.814	0.154	-0.338	0.242	0.089
<i>n</i> C ₁₉ ¹³ C	0.869	0.358	-0.226	0.006	-0.101
<i>n</i> C ₂₀ ¹³ C	0.754	0.412	-0.377	-0.066	-0.216
<i>n</i> C ₂₁ ¹³ C	0.744	0.481	-0.298	0.013	-0.242
<i>n</i> C ₂₂ ¹³ C	0.798	0.366	-0.227	0.117	-0.342
<i>n</i> C ₂₃ ¹³ C	0.854	0.267	-0.124	0.123	-0.310
<i>n</i> C ₁₂ ² H	0.370	-0.386	-0.752	-0.077	0.191
<i>n</i> C ₁₃ ² H	0.794	-0.372	-0.093	-0.393	-0.016
<i>n</i> C ₁₄ ² H	0.876	-0.276	0.057	-0.324	-0.043
<i>n</i> C ₁₅ ² H	0.916	-0.287	0.085	-0.165	0.002
<i>n</i> C ₁₆ ² H	0.869	-0.229	0.175	-0.365	0.002
<i>n</i> C ₁₇ ² H	0.922	-0.224	0.131	-0.246	0.010
Pris ² H	0.635	-0.378	0.316	0.026	0.008
<i>n</i> C ₁₈ ² H	0.686	-0.315	0.563	-0.061	-0.026
Phy ² H	0.377	-0.427	-0.570	0.444	0.206
<i>n</i> C ₁₉ ² H	0.572	-0.607	0.333	0.228	0.130
<i>n</i> C ₂₀ ² H	0.673	-0.576	-0.098	0.266	0.029
<i>n</i> C ₂₁ ² H	0.579	-0.597	0.001	0.435	0.085
<i>n</i> C ₂₂ ² H	0.655	-0.549	0.019	0.325	-0.034
<i>n</i> C ₂₃ ² H	0.672	-0.305	0.488	0.107	-0.142

Results are based on the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of the alkane compounds.

^a5 components extracted.

hold time of 1 min, temperature ramp at 10°C min to 300°C, and final hold time 4 min.

Instrumental drift during individual sample analysis (~30 min) was corrected by injecting multiple pulses of monitoring gas (CO₂ or) at the beginning and end of each sample run. While instrumental drift during a batch sample was corrected by injecting a standard mixture (containing seven *n*-alkanes whose $\delta^{13}\text{C}$ and $\delta^2\text{H}$ had been previously determined by bulk IRMS) every six samples. The instrument analytical precision for compound specific $\delta^{13}\text{C}$ and $\delta^2\text{H}$ analysis was determined to be <0.3 and 3‰, respectively, based on long-term repeatability of a control sample. Isotopic compositions of each alkane were expressed as δ values per mil (‰) deviation relative to isotopic standard reference materials:

$$\delta = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \quad (1)$$

where $R = {}^{13}\text{C}/{}^{12}\text{C}$ or ${}^2\text{H}/{}^1\text{H}$. The $\delta^{13}\text{C}$ were reported relative to the Vienna Pee Dee Belemnite (VPDB), while $\delta^2\text{H}$ values were reported relative to the Vienna Standard Mean Ocean Water (VSMOW) standard. The H_3^+ factor was determined daily using the standard hydrogen gas introduced through the interface. The H_3^+ is a byproduct species formed during ion-molecule reaction which can interfere with the measurement of HD, so correction was required for H isotope determination. The mass spectrometer was tuned to ensure that the H_3^+ factor was less than 10 nA/ppm and the daily variability was <0.1.

ISOTOPIC COMPOSITION OF ALKANES IN DIESEL FUEL

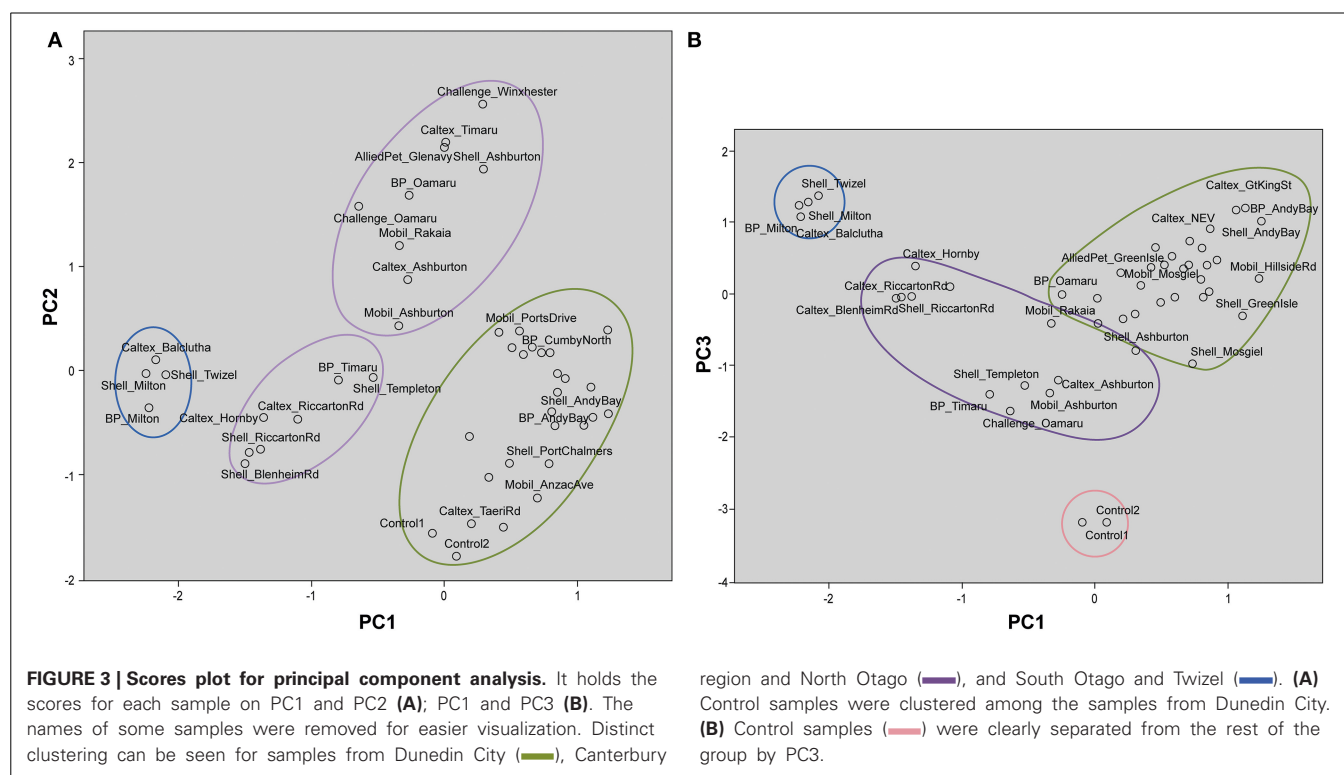
Diesel fuel is a complex mixture of hydrocarbon molecules derived from petroleum crude oil and may contain thousands

of individual compounds, most with carbon numbers between 9 and 23. This complexity can cause analytical problems in gas chromatography such as peak separation and baseline resolution, even more so with GC-IRMS as true compound-specific isotopic analysis requires baseline resolution and no co-eluting peaks. Hence, it is important to note that the measured stable isotope ratios across selected peaks may therefore include some underlying co-eluting material and are not entirely specific for individual compounds. However, the data in this study remain forensically relevant as part of an “isotopic fingerprint pattern.” The alkane compounds which were able to be reliably quantified and yielded reproducible isotopic values using a GC-IRMS system in this study started with $n\text{C}_{12}$ and ended with $n\text{C}_{23}$.

DATA TREATMENT USING STATISTICAL METHODS

All mathematical and statistical computations were made using Excel 2007 (Microsoft Office®), SigmaPlot 11.0 (Systat Software Inc.®) and SPSS 16.0 (IBM®). Multivariate statistical analysis of the stable isotope data was performed using PCA and HCA.

PCA is a mathematical procedure that converts the possibly correlated original variables into new linearly uncorrelated variables, called the principal components (PCs). This technique also reveals the internal structure of the data and finds the indices which best explains the variance in the data set. PCA also provides the most meaningful parameters which interpret the whole data set, thus reducing the dimensionality of the transformed data and summarize the statistical correlation among constituents with minimum loss of original information (Kazi et al., 2009). For the purpose of this study, PCA was used as an exploratory



technique to group samples with similar isotopic compositions in PC space. The outcomes of the analysis can be visualized using scores plot which illustrate the tendency of a sample grouping. The contribution of certain characteristics which is called patterns or loadings of the data set will determine the position of each sample in the scores space (Pasadakis et al., 2008).

HCA is an analysis that builds a hierarchy of clusters by measuring either the distance or the similarity between the objects

to be clustered. It is normally used when there are no *a priori* hypotheses. The hierarchical agglomerative clustering or the “bottom up” method is the preferred approach for applying this technique. Briefly, it builds the hierarchy from the individual elements by progressively merging clusters. The results of HCA are normally illustrated using a dendrogram (tree diagram). The dendrogram summarizes the clustering process, showing the number of clusters (number criterion) and indicating their proximity in

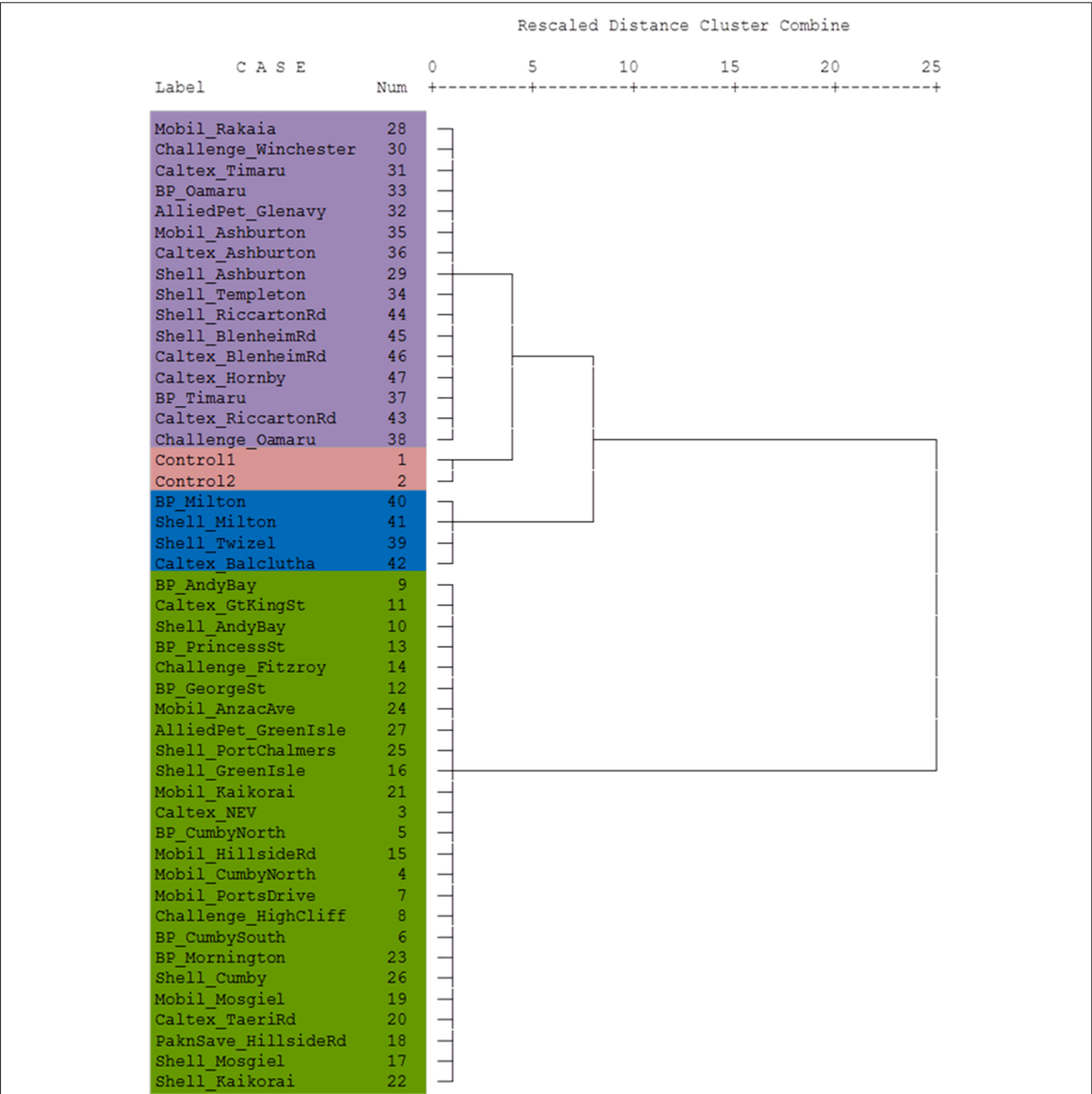


FIGURE 4 | Dendrogram using Ward’s linkage method showing cluster relationship between Control and South Island of New Zealand. Lengths of vertical lines represent statistical difference

between multivariate components in each sample. The different colors reflect the different areas where the samples were obtained (see Figure 1).

space (distance criterion), thus reducing the dimensionality of the original data. To analyze the data set in this study, HCA was performed using Ward's linkage method. The Ward's method minimizes the total within-cluster variance, and the cluster distances are defined by the squared Euclidean distances as a measure of similarity.

RESULTS AND DISCUSSION

CARBON ISOTOPE ANALYSIS

The ranges of carbon isotope ratios of each individual alkane in Control, GKT and South Island of New Zealand samples are illustrated in **Figure 2A**. The mean value of pristane was lighter (-30.1‰) when compared with phytane and the rest of the alkanes. **Table 1** shows the statistical analysis of the $\delta^{13}\text{C}$ values of individual alkanes in the same set of samples. The range of carbon isotope compositions of the alkanes within these diesel samples (-33.3 to -25.8‰) was comparable to the values determined in previous work (Mazeas et al., 2002; Sun et al., 2005). These compounds show a broad range of carbon isotopic ratios, the greatest being observed for phytane (-33.3 to -28.3‰), $n\text{C}_{23}$ (-31.7 to -27.2‰) and pristane (-33.3 to -29.2‰). There was no significant difference in $\delta^{13}\text{C}$ values of odd and even numbered alkanes.

HYDROGEN ISOTOPE ANALYSIS

Table 1 presents the $\delta^2\text{H}$ values of individual alkanes in Control, GKT and South Island of New Zealand samples. The largest range of $\delta^2\text{H}$ was found in $n\text{C}_{12}$ (-100.0 to -45.5‰), $n\text{C}_{15}$ (-96.2 to -58.1‰) and $n\text{C}_{17}$ (-97.6 to -59.7‰). The wide range of hydrogen isotopic values for these alkanes was in agreement with previous published result (Li et al., 2001). The ranges in $\delta^2\text{H}$ values of each of the alkane compounds are illustrated in **Figure 2B**. The findings from the analysis of $\delta^2\text{H}$ values revealed similar patterns as observed in the $\delta^{13}\text{C}$.

MULTIVARIATE STATISTICAL ANALYSIS

The forensic evidence provided by stable isotope analysis is circumstantial in that when two samples are indistinguishable that supports, but does not prove, the hypothesis that they are related. The likelihood that they are actually related can only be estimated by measuring control samples and determining how likely two samples could have identical isotopic character by chance. In the present context it is important to establish whether samples obtained from the same source region have similar isotopic characteristics and are distinctive from samples obtained from other source regions. Therefore, it is imperative that control samples be subject to a population study in order for analysts to make an informed assessment of the likelihood. The control samples used here were commercially available diesel samples obtained from around the South Island of New Zealand (Muhammad et al., 2013) plus the Control samples supplied by NZ Police. The stable isotope ratios of individual alkanes from all the diesel samples were subjected to PCA and HCA. The information obtained from the statistical analyses was used to associate and differentiate diesel samples based on $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values. The first principal component (PC1) described 50.4% of the variance in the data. The second principal component (PC2) described an additional

19.1% of the variance while the third principal component (PC3) explained further 9.6% of the data variability. Thus, 79.1% of the total variance was explained by the first three components.

Table 2 shows the component matrix which listed $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values as variables and their contribution to the variance in the data set. Component matrix 1 shows the $\delta^{13}\text{C}$ values of the alkane compounds contributed evenly with large positive coefficients. This implies that they have equal weighting in the interpretation of the scores for PC1. Likewise, $\delta^2\text{H}$ values of the alkane compounds show positive coefficients for the same principal component. As for PC2, $\delta^{13}\text{C}$ still shows positive coefficients for all the compounds. On the other hand, $\delta^2\text{H}$ values of the compounds for this component show negative correlations to $\delta^{13}\text{C}$ although both variables may have equal weighting and contribution to the interpretation of the scores. In component matrix 3, the top contributors to the scores were the $\delta^2\text{H}$ values of $n\text{C}_{12}$ and $n\text{C}_{18}$ with highly negative coefficients.

Table 3 | Component matrix which shows variables and their contribution to the variance in the data set of Control, GKT, and South Island of New Zealand samples.

	Component ^a				
	1	2	3	4	5
$n\text{C}_{12}^{13}\text{C}$	0.709	-0.199	-0.486	0.012	0.008
$n\text{C}_{13}^{13}\text{C}$	0.686	0.009	-0.437	-0.064	0.135
$n\text{C}_{14}^{13}\text{C}$	0.751	0.202	-0.303	-0.176	-0.148
$n\text{C}_{15}^{13}\text{C}$	0.254	0.364	-0.626	-0.241	0.435
$n\text{C}_{16}^{13}\text{C}$	0.335	0.644	-0.308	-0.071	0.527
$n\text{C}_{17}^{13}\text{C}$	0.481	0.078	-0.456	0.226	0.544
Pris ¹³ C	0.738	0.541	0.202	-0.203	0.180
$n\text{C}_{18}^{13}\text{C}$	0.863	0.172	-0.312	0.045	-0.086
Phy ¹³ C	0.663	0.603	0.249	-0.243	0.015
$n\text{C}_{19}^{13}\text{C}$	0.878	0.364	-0.102	-0.034	-0.151
$n\text{C}_{20}^{13}\text{C}$	0.773	0.452	-0.144	-0.002	-0.273
$n\text{C}_{21}^{13}\text{C}$	0.757	0.050	-0.404	0.151	-0.302
$n\text{C}_{22}^{13}\text{C}$	0.774	0.069	-0.284	0.370	-0.219
$n\text{C}_{23}^{13}\text{C}$	0.812	0.027	-0.185	0.419	-0.133
$n\text{C}_{12}^2\text{H}$	0.200	0.724	0.558	0.054	-0.218
$n\text{C}_{13}^2\text{H}$	0.715	0.049	0.431	-0.264	-0.203
$n\text{C}_{14}^2\text{H}$	0.810	-0.448	0.201	-0.148	-0.144
$n\text{C}_{15}^2\text{H}$	0.788	-0.561	0.138	-0.065	-0.057
$n\text{C}_{16}^2\text{H}$	0.777	-0.555	0.080	-0.149	-0.101
$n\text{C}_{17}^2\text{H}$	0.799	-0.571	0.076	-0.025	-0.076
Pris ² H	0.487	-0.439	0.105	-0.529	0.208
$n\text{C}_{18}^2\text{H}$	0.491	-0.792	-0.011	0.049	0.262
Phy ² H	0.215	0.674	0.552	-0.370	0.061
$n\text{C}_{19}^2\text{H}$	0.447	-0.565	0.455	-0.027	0.384
$n\text{C}_{20}^2\text{H}$	0.539	0.000	0.667	-0.230	0.159
$n\text{C}_{21}^2\text{H}$	0.389	-0.020	0.643	0.463	0.222
$n\text{C}_{22}^2\text{H}$	0.457	0.130	0.644	0.479	0.158
$n\text{C}_{23}^2\text{H}$	0.382	0.210	0.261	0.634	0.199

Results are based on the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of the alkane compounds.

^a5 components extracted.

These interpretations are visualized on the scores plot in **Figure 3A** (PC1 vs. PC2) and **Figure 3B** (PC1 vs. PC3). **Figure 3A** shows two distinct clusters with South Otago and Twizel samples in one group and Dunedin City in another. The Control samples are grouped in the same cluster as the Dunedin City samples. The samples obtained from the Canterbury and North Otago appears to be separated into 2 clusters by PC2. However, in **Figure 3B** several clusters are apparent comprising South Otago and Twizel samples in one group, Dunedin City in another and Canterbury region and North Otago samples in a group of its own in the middle of the plot. The Control samples were clearly separated from the other groups due to the substantial contribution by PC3.

The dendrogram presented in **Figure 4** shows that the Control diesel samples were subtly correlated with the samples from Canterbury region and North Otago samples. South Otago and Twizel samples were grouped together and showed moderate correlation with the Control, Canterbury region and North Otago samples. The dendrogram also shows clear separation of Dunedin City samples from the rest of the group as illustrated in the large jump of the linkage which indicates the clusters are far apart (read to the right of the dendrogram to see the clusters).

The statistical analyses on the subtle differences in $\delta^{13}\text{C}$ and $\delta^2\text{H}$ of alkanes enabled clear discrimination of the Control samples from the other South Island samples. Following this observation, similar statistical treatments were carried out for Control and GKT samples, together with the South Island of New Zealand samples.

The PCA analysis resulted in a first principal component (PC1) that described 40.9% of the variance in the data. The second principal component (PC2) described an additional 17.9% of the variance while the third principal component (PC3) explained

further 14.8% of the data variability. Thus, 73.6% of the total variance was explained by the first three components.

Table 3 shows the component matrix with $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values as variables and their contribution to the variance in the data set comprised of the Control, GKT and South Island of New Zealand samples. Component matrix 1 shows the $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values of the alkane compounds contributed evenly with highly positive coefficients for most of the compounds. This implies that they have equal weighting in the interpretation of the scores for PC1. For PC2, $\delta^2\text{H}$ values of the compounds were mostly negatively correlated to the $\delta^{13}\text{C}$ variables with more compounds showing higher weightage and contributed more to the interpretation of the scores. In component matrix 3, an opposing trend for $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values was seen with $\delta^{13}\text{C}$ variables showing negative correlations to that of $\delta^2\text{H}$ variables.

These interpretations from PCA were visualized on the scores plots in **Figures 5A,B**. **Figure 5A** shows distinct clusters comprised of South Otago and Twizel samples in one group and Dunedin City samples in another. The Canterbury region and North Otago samples showed moderate clustering in the middle of the scores plot. GKT 107/1, GKT 107/2, and GKT 108 were separated from the rest of the diesel samples by PC2 whilst Control and other GKT samples fell in the cluster of Canterbury region and North Otago samples. When PC1 was plotted against PC3 (**Figure 5B**), similar observations were seen for South Otago and Twizel and Dunedin City clustering in **Figure 5A**. The primary difference is that Control samples are now grouped together with GKT 106, GKT 202/1, and GKT 202/2. Clearly, this pattern was mainly due to the contribution from PC3. However, some of the GKT samples were seen to have similarities with that of Dunedin City samples as scores plot showed moderate clustering between them.

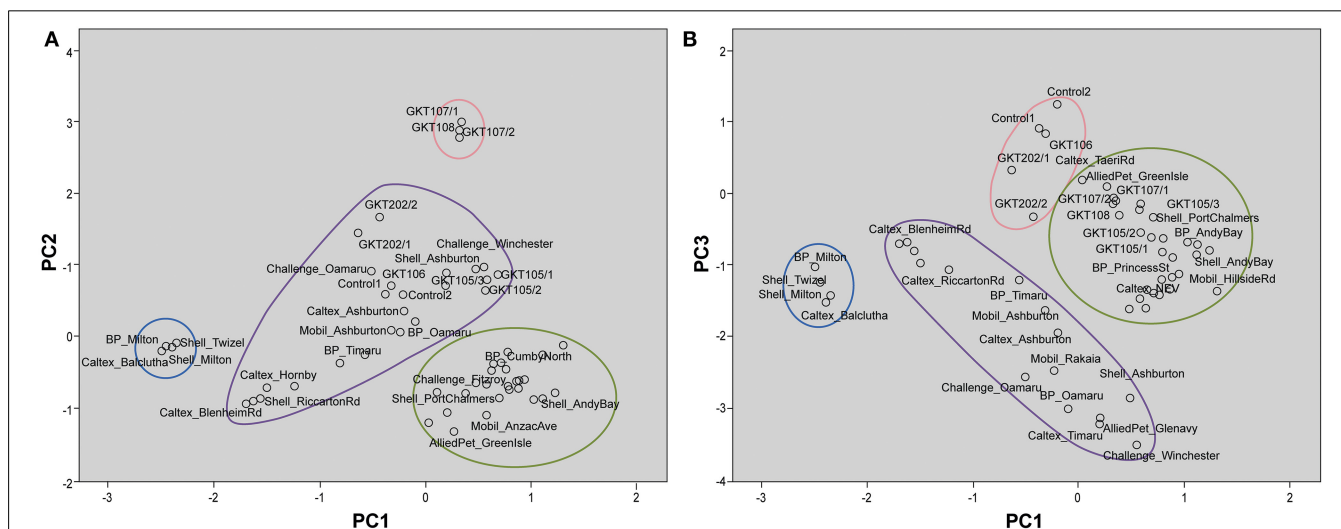


FIGURE 5 | Scores plot for principal component analysis. It holds the scores for each sample on PC1 and PC2 (**A**); PC1 and PC3 (**B**). The names of some samples were removed for easier visualization. (**A**) Distinct clustering can be seen for samples from Dunedin City (—), Canterbury region and North Otago (—), and South Otago and Twizel (—). GKT 107/1, GKT 107/2, and GKT 108 (—) were separated from the rest of the groups by PC2 whilst

Control and other GKT samples were grouped in the cluster of Canterbury region and North Otago. (**B**) Distinct clustering can be seen for samples from Dunedin City (—), Canterbury region and North Otago (—), and South Otago and Twizel (—). PC3 showed substantial contribution in separating Control, GKT 106, GKT 202/1, and GKT 202/2 samples (—) from the rest of the data set.

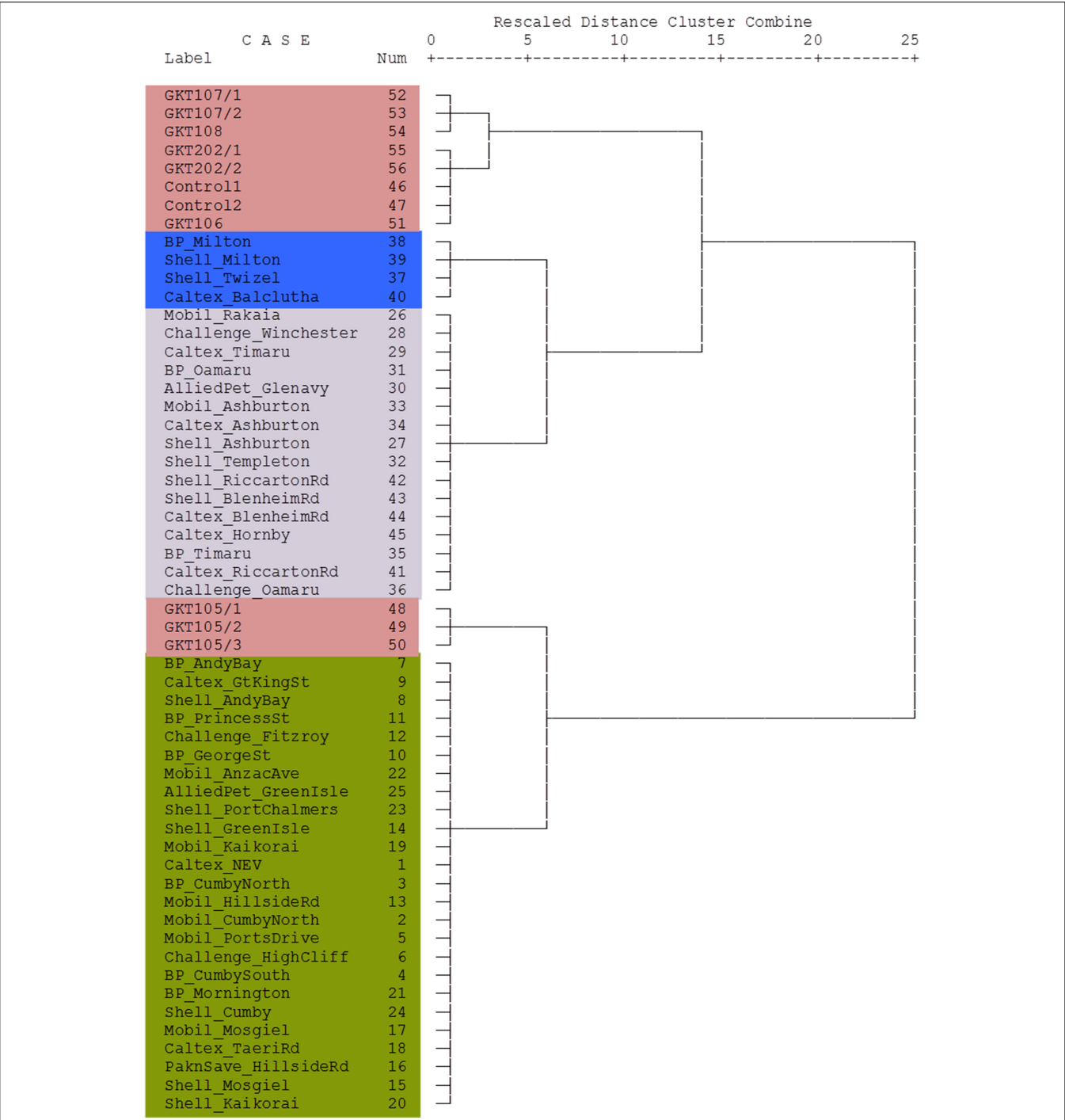


FIGURE 6 | Dendrogram using Ward's linkage method showing cluster relationship between Control, GKT, and South Island of New Zealand samples. Lengths of vertical lines represent statistical

difference between multivariate components in each sample. The different colors reflect the different areas where the samples were obtained (see **Figure 1**).

The stable isotope data of the diesel samples involved in the theft case were also analyzed using cluster analysis to see their association with the diesel samples from the South Island of New Zealand. This relationship was presented in a dendrogram in **Figure 6**. Here the Blenheim diesel samples are grouped into small clusters with samples under the same denominations such

as GKT 107 samples and GKT 108, classed together. Likewise, the GKT 106 sample found to be highly correlated with GKT 202 samples and Control diesels. Additionally, except for GKT 105 samples, the rest of the Blenheim diesels were closely related and showed only a small amount of variation between them. GKT 105 diesels were more closely correlated with the samples from

Dunedin City service stations than the samples from Blenheim, as seen by the large jump of the linkage between them. Similar discrimination is observed for the rest of the commercial diesel samples with each of the group classed together due to the high correlation between them.

CONCLUSIONS

The forensic discrimination of diesel fuels involved in a theft case utilizing stable isotope fingerprint of hydrocarbon indices was achieved. There were variations in the stable isotopic compositions of alkanes within the diesel samples that can be used to differentiate them. Additionally, a large population of commercial diesel fuels obtained from various areas in the South Island of New Zealand was included in the data set to rule out statistical coincidence as well to put the data into context thus providing an informed assessment of the analysis.

Discrimination of the diesel samples into groups was evident by comparing the subtle differences in the stable isotope values of the alkane compounds using multivariate statistical analyses such as PCA and HCA. The main conclusions from these analyses are:

- PCA showed that the Control diesel samples were strongly correlated with GKT 106 and GKT 202 samples and moderately correlated with the other GKT samples.
- Diesel samples that were grouped together based on PCA also showed high correlation using HCA.
- However, GKT 105 samples were shown to be grouped separately from the other Blenheim samples using HCA.
- PCA and HCA both highlighted the association of Control samples with that of GKT 106, GKT 107, GKT 108, and GKT 202 samples.
- In our opinion, the most likely explanation is that these diesels share a common history.

In summary, this paper provided some context on the Blenheim diesel theft case. The Control samples were found to be chemically related to samples found in possession of the suspect, i.e., they were indistinguishable by measurements of their stable isotope fingerprint. That they were distinguishable from the 45 additional samples collected from around the South Island and that the South Island samples were able to be grouped according to source provided support for the prosecution hypothesis that the samples in possession of the suspect had originated from the theft. When this evidence was presented in court the accused changed their plea to guilty and was convicted of the theft.

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Protein S-nitrosylation: specificity and identification strategies in plants

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The role of nitric oxide (NO) as a major regulator of plant physiological functions has become increasingly evident. To further improve our understanding of its role, within the last few years plant biologists have begun to embrace the exciting opportunity of investigating protein S-nitrosylation, a major reversible NO-dependent post-translational modification (PTM) targeting specific Cys residues and widely studied in animals. Thanks to the development of dedicated proteomic approaches, in particular the use of the biotin switch technique (BST) combined with mass spectrometry, hundreds of plant protein candidates for S-nitrosylation have been identified. Functional studies focused on specific proteins provided preliminary comprehensive views of how this PTM impacts the structure and function of proteins and, more generally, of how NO might regulate biological plant processes. The aim of this review is to detail the basic principle of protein S-nitrosylation, to provide information on the biochemical and structural features of the S-nitrosylation sites and to describe the proteomic strategies adopted to investigate this PTM in plants. Limits of the current approaches and tomorrow's challenges are also discussed.

Keywords: nitric oxide, S-nitrosylation, post-translational modifications, plants, signaling, biotin switch technique

INTRODUCTION

In animals, the free radical nitric oxide (NO) serves as an important messenger of intra- and extracellular pathways and regulates a myriad of physiological processes (Schmidt and Walter, 1994; Mustafa et al., 2009). For instance, its involvement as a modulator of vascular tone, neurotransmission, platelet aggregation, reproductive systems, and immune responses are widely recognized. The involvement of NO as a physiological mediator is not restricted to animals. Notably, results of intensive investigations achieved over the past 15 years indicate that NO also regulates diverse biological processes in plants. Key roles for NO have been demonstrated in seed dormancy, embryogenic cell formation, root development and gravitropic bending, flowering, stomatal closure, growth regulation of pollen tubes, nutrition and particularly iron homeostasis, immunity, and adaptive responses to various abiotic stresses (Wilson et al., 2008; Wendehenne et al., 2014; Yu et al., 2014). Comprehensive studies were undertaken in order to clarify the molecular mechanisms underlying NO functions in plants. Clearly, the cellular activities of NO are numerous and complex: NO operates through reactive oxygen species (ROS) and classical second messengers including Ca^{2+} and cGMP, regulates the activity of metabolic and signaling proteins such as protein kinases, impacts the organization of cytoskeleton and actin-dependent vesicle trafficking and modulates the expression of numerous genes involved in essentially all cellular functions (Besson-Bard et al., 2009; Kaspricz et al., 2009; Leitner et al.,

2009; Gaupels et al., 2011; Jeandroz et al., 2013; Trapet et al., 2014). Furthermore, cross-talks between NO and key hormones including auxin, abscisic acid, salicylic acid (SA), jasmonic acid, ethylene, and cytokinins have been reported (Lamattina et al., 2003; Wilson et al., 2008; Terrile et al., 2012; Feng et al., 2013; Mur et al., 2013). Another issue of investigations concerned the origins of NO produced by plant cells. Nitrite is unquestionably a main substrate for NO synthesis through both non-enzymatic and enzymatic processes involving nitrate reductase (Gupta et al., 2011). A body of arguments also suggests that plants could possess an enzyme which, similarly to animal nitric oxide synthases (NOS), could use L-arginine as a substrate (Corpas et al., 2009). This latter possibility is still debated.

How NO governs cellular reactions at the molecular level has been and is still the subject of important researches in animal biology. The current available data illustrate that NO actions, as well as those of certain NO-derived species, depend on chemical modifications of proteins. Three main processes are now recognized: nitration referring to the binding of a NO_2 group to Tyr residues, metal- and S-nitrosylation referring to the binding of a NO group to a transition metal or a Cys residue, respectively (Mannick and Schonhoff, 2002). S-nitrosylation has emerged as an important NO-dependent post-translational modification (PTM) of proteins regulating a large variety of cellular functions and signaling events (Hess et al., 2005; Gould et al., 2013). In plants, the search and functional analysis of S-nitrosylated proteins has also grown

substantially this last decade. These investigations resulted in original data and S-nitrosylation is now accepted as a cell signaling mechanism with important functional implication in various plant physiological processes (Lindermayr and Durner, 2009; Spadaro et al., 2010; Astier et al., 2012b). In the present review, we have considered the question of S-nitrosylation in plant cells, with a particular emphasis on proteomic approaches for the identification of S-nitrosylated proteins either by proteomic approaches or by algorithm-based SNO site identification.

BASIC KNOWLEDGE ABOUT PROTEIN S-NITROSYLATION IN ANIMALS

S-nitrosylation is a reversible covalent chemical reaction in which a NO moiety is coupled to a critical Cys thiolate (S^-) on a target protein (Martinez-Ruiz et al., 2011; Gould et al., 2013). This process leads to the formation of an S-nitrosothiol (SNO) and is mediated by NO through a thiyl radical recombination by higher oxides of NO such as dinitrogen trioxide (N_2O_3) or nitrosonium cation (NO^+), by metal-NO complexes or low molecular weight S-nitrosothiols including S-nitrosocysteine (CysNO) and the major physiological NO donor nitrosoglutathione (GSNO) (Hess et al., 2005; Hill et al., 2010; Smith and Marletta, 2012). Several S-nitrosylated proteins including SNO-hemoglobin, -thioredoxin

(Trx), -caspase 3, or -glyceraldehyde-3-phosphate dehydrogenase (GAPDH) can also catalyze the transfer of their NO group to an adjacent thiol of a binding partner; a mechanism referred as transnitrosylation (**Figure 1**) (Kornberg et al., 2010; Nakamura and Lipton, 2013; Sengupta and Holmgren, 2013). This PTM triggers conformational changes of proteins, can affect their activities, sub-cellular localization and interactions with partners. Over the past 20 years, thanks to the emergence of dedicated approaches such as the biotin-switch technique (BST, see below), over 3000 protein candidates for S-nitrosylation under normal and/or pathological conditions have been identified in animal cells (Hess and Stamler, 2012). A database (dbSNO 2.0) centralizing S-nitrosylated proteins collected from the literature is available at dbSNO 2.0 <http://dbSNO.mbc.nctu.edu.tw> (Chen et al., 2014). These proteins cover a wide range of cellular functions and include, amongst others, receptors, ion channels, signaling proteins, metabolic enzymes, proteases, chaperones, and structural proteins (as examples see Seth and Stamler, 2011; Nakamura et al., 2013; Ben-Lulu et al., 2014). Denitrosylation, the removal of NO from Cys residues, has also emerged as a key mechanism regulating protein activities, protein-protein interactions and more generally signaling (Martinez-Ruiz et al., 2013). For instance, certain proteins constitutively S-nitrosylated such as the pro-form

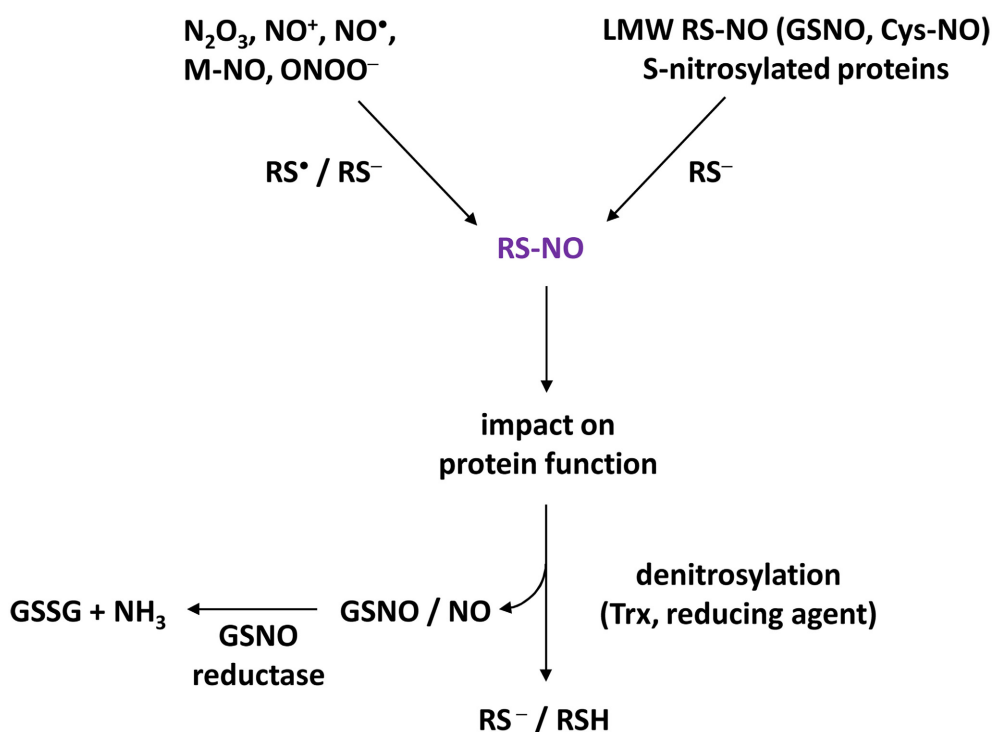


FIGURE 1 | Basic concept of S-nitrosylation. Higher oxides of NO (such as N_2O_3 , NO^+), NO as a free radical ($\text{NO}^\bullet$), metal-NO complexes (M-NO) or NO derivatives such as peroxynitrite (ONOO^-) are able to trigger S-nitrosylation by interacting with Cys thiolate (RS^-) or thiyl ($\text{RS}^\bullet$) of target proteins, thus leading to S-nitrosothiols (RS-NO). Furthermore, S-nitrosylation can be mediated by transnitrosylation through S-nitrosylated proteins and low molecular weight (LMW) S-nitrosothiols such as nitrosoglutathione (GSNO) or S-nitrosocysteine (Cys-NO). S-nitrosylation

impacts the function of target proteins by affecting their activities (activation, inhibition), their subcellular localizations and interactions with partners. S-nitrosylation is a reversible process mediated by reducing compounds such as GSH but also by thioredoxins (Trx). Denitrosylation could therefore lead to the formation of GSNO which, in turn, is metabolized into glutathione disulfide (GSSG) and ammonia by GSNO reductase (GSNO reductase). Therefore, Trx and GSNO reductase appear to be critical for SNO homeostasis.

of caspase 3 become activated upon denitrosylation (Mannick et al., 1999). This regulation also constitutes a powerful mechanism protecting cells from nitrosative stresses (Benhar et al., 2009). It involves non-enzymatical as well as enzymatical processes in which Trx and GSNO reductase catalyzing the reduction of GSNO, and therefore negatively regulating cellular levels of SNO, seem to play a major role (**Figure 1**) (Anand and Stamler, 2012; Martinez-Ruiz et al., 2013).

An ongoing issue concerns the selectivity and specificity of S-nitrosylation for certain proteins and Cys residues. Two major parameters have been highlighted. The first concerns the co-localization of target proteins with NOS. The presence of both the NO source and the S-nitrosylation substrate within discrete sub-cellular domains favors high local concentrations of nitrosylating species and the targeting of NO to specific Cys residues (Stamler et al., 2001; Mannick and Schonhoff, 2002; Kone et al., 2003; Martinez-Ruiz et al., 2011). This spatial and temporal confinement allows a tight regulation of NO signaling. The second is related to the reactivity of certain Cys residues. Structural, biochemical, and bioinformatics approaches have been applied in order to define common features of the Cys residues of interest. The latter seem to be positioned in a favorable chemical context increasing their nucleophilicity and therefore thiol ionization (Lane et al., 2001). In this regard, the proximity of basic and aromatic residues emerged as a possible factor promoting S-nitrosylation (Derakhshan et al., 2007). This feature is not exclusive and other parameters have been pointed out such as the occurrence of an acid–base motif within 6–8 Å of the modified Cys (Doulias et al., 2010) or more distant but facilitating trans-nitrosylation (Marino and Gladyshev, 2010), the presence of hydrophobic residues allowing formation of efficient nitrosylating species in the vicinity of the Cys residue (Lane et al., 2001) and the localization of the Cys residue in a solvent-accessible surface of the protein (Marino and Gladyshev, 2012). The denitrosylation rate is probably another parameter of importance. In a recent study, Cheng et al. (2014) investigated the structural and biochemical features of S-nitrosylated Cys residues based on 213 structures of S-nitrosylated proteins for which structural information was available. According to previous studies, compared to non-S-nitrosylated Cys residues, the S-nitrosylated ones have a lower pK_a and appeared to be more flexible and preferentially surrounded within 6 Å by basic residues which could enhance their deprotonation as well as stabilize their deprotonated state. A lower abundance of bulky residues (such as Phe, Tyr, Arg, and Leu) in the neighboring region within 8 Å of the Cys residues undergoing S-nitrosylation was also noted, suggesting that steric hindrance could be a disadvantage for the process of S-nitrosylation. Another feature listed in this study was a reduced frequency of Cys residues around the S-nitrosylation site, especially when the inter-residue distance was less than 3.5 Å. According to the authors, this structural particularity might reduce the competition for oxidant agents in the process of S-nitrosylation.

In sum, although partially conserved features of the nitrosylation sites emerged, these analyses have not yielded a dominant consensus motif. As pointed out by Smith and Marletta (2012), this statement is probably due to the various mechanisms

leading to SNO formation. Web-servers dedicated to the prediction of S-nitrosylation sites have been developed. These programs are based on S-nitrosylation sites identified experimentally and, depending on the servers, features such as the identity of the flanking residues, solvent accessibility and the protein secondary and tertiary structures are taken into account for screening the Cys residue of interest. They are freely accessible: GPS-SNO (<http://sno.biocuckoo.org/>, Xue et al., 2010), SNOsite (<http://csb.cse.yzu.edu.tw/SNOsite/>, Lee et al., 2011), dbSNO 2.0 (<http://dbSNO.mbc.nctu.edu.tw>, Lee et al., 2012; Chen et al., 2014), iSNO-PseAAC (<http://app.aporc.org/iSNO-PseAAC/>, Xu et al., 2013), PSNO (<http://59.73.198.144:8088/PSNO/>, Zhang et al., 2014). Recently, Huang et al. (2014) proposed a new web-server (<http://www.zhni.net/snopred/index.html>) for the prediction of S-nitrosylation in which additional parameters such as evolutionary conservation and disorder status of amino-acid residues were also included.

S-NITROSYLATION IN PLANTS: BRIEF INSIGHTS

The introduction of the BST described below has served as an impetus for screening for S-nitrosylated proteins in plants. Proteomic identification with this method was first achieved in plant tissues, cell suspensions or protein extracts exposed to nitrosylating agents, mainly GSNO. Additional studies also searched for proteins constitutively S-nitrosylated in plant cells or undergoing S-nitrosylation under physiological conditions including hormone signaling and responses to pathogens, PAMPs (Pathogen-Associated Molecular Patterns) and abiotic stresses such as high light, salinity, cold, and heavy metals (Spoel and Loake, 2011; Mengel et al., 2013; Romero-Puertas et al., 2013; Puyaubert and Baudouin, 2014; Trapet et al., 2014; Yu et al., 2014). Currently, well over a 100 proteins susceptible to S-nitrosylation have been identified. Amongst those, few have been thoroughly studied and confirmed to undergo S-nitrosylation *in vivo* (Astier et al., 2012b; Kovacs and Lindermayr, 2013; Skelly and Loake, 2013). Supplementary Table 1 lists several of these proteins and highlights the incidence of NO on their structure/function at the protein and, when investigated, at the physiological levels. What can we conclude about these pioneer studies? First, as reported in animals, S-nitrosylation appears to be implicated in the regulation of a wide array of protein functions and cell activities, particularly signaling, redox balance, metabolism, protein quality control and transcription. Second, the impact of this PTM differs according to the target protein: it promotes conformational changes, facilitates its oligomerization through the formation of disulfide linkages between monomers, inhibits the binding of cofactors such as ATP or NADPH or affects their activities by interacting with catalytic Cys residues. Third, enzymatic denitrosylation mechanisms occur through Trx and GSNO reductase (Malik et al., 2011; Kneeshaw et al., 2014).

In recent investigations, Kovacs and Lindermayr (2013) and Chaki et al. (2014) focused on the structural features of S-nitrosylation sites of plant proteins. For this purpose, the authors performed a computational prediction of S-nitrosylation sites of plant proteins experimentally found as S-nitrosylated and for which the corresponding Cys residue(s) have been identified by MS analysis. The programs GPS-SNO, SNOsites,

and iSNO-PseAAC were used. The programs all predicted S-nitrosylation sites in those proteins. However, the number of Cys residues and their identity differed according to the computational programs. The GPS-SNO best matched the MS analysis. This performance confirmed a previous work demonstrating that amongst 485 potentially S-nitrosylated proteins collected from PubMed, the GPS-SNO program predicted at least one putative S-nitrosylation sites in 74% of them (Xue et al., 2010). Another in-depth analysis of the structural features of plant S-nitrosylated proteins was provided by Fares et al. (2011). Using the Motif-X-algorithm (<http://motif-x.med.harvard.edu/>) extracting overrepresented patterns from a sequence data set, these authors screened for motifs flanking S-nitrosylated Cys-residues (± 10 residues) amongst 53 proteins found to be constitutively regulated by this PTM in *Arabidopsis thaliana*. Three motifs involving hydrophobic residues were found (A-X(9)-C, C-X(6)-G, and C-X(2)-I). Interestingly, at least one of these motifs was detected in about half of 121 proteins that were previously identified as putatively S-nitrosylated in other studies. Amongst the selected proteins, 38 sites for S-nitrosylation were also predicted using the program SNOsite.

We applied this series of prediction to CDC48 (Cell Division Cycle 48), a chaperone-like AAA+ ATPase found to be constitutively S-nitrosylated in *A. thaliana* on Cys-109 (Fares et al., 2011) and on Cys-526 upon immune stimulation in tobacco (Astier et al., 2012a). In tobacco, Cys 110 (Cys 109 in *A. thaliana*), Cys-526 and Cys-664 were also found to undergo S-nitrosylation *in vitro* following the exposure of the recombinant protein with GSNO (Astier et al., 2012a). A case study for Cys-109 of

A. thaliana CDC48 is shown **Figure 2**. In the primary sequence, Cys-109 is surrounded by two β sheets and its solvent accessibility appears to be low (**Figures 2A,B**). Furthermore, this residue locates in a region flanking with acidic and basic residues. Abundance of these residues around the Cys residues of interest is confirmed by the search for statistically significant conserved S-nitrosylation motifs (**Figure 2C**). Furthermore, as discussed above (Cheng et al., 2014), no Cys residue was found in the region flanking Cys-109.

IDENTIFICATION OF PLANT S-NITROSYLATED PROTEINS: METHODOLOGICAL ASPECTS

THE BIOTIN-SWITCH TECHNIQUE

The BST, initially developed by Jaffrey et al. (2001) (see also Jaffrey and Snyder, 2001) provides an efficient methodological tool for identifying S-nitrosylated proteins. In particular, this method greatly avoids the constraint of the inherent lability of protein S-NO groups. Basically, the BST involves three steps (**Figure 3**). In the first step, the blocking step, proteins extracted from tissues, cultured cells, purified organelles or recombinant proteins are incubated at 50°C with a thiol-reacting reagent, mainly methyl-methane thiosulfonate (MMTS), in the presence of sodium dodecyl sulfate (SDS). The combination of moderate heat and SDS favors protein denaturation and thus increases the accessibility of protein free thiols to the thiol-reacting reagent. This step allows the S-methylthiolation and therefore the blocking of free Cys thiols. In the second step, after removing the excess of MMTS, S-nitrosylated Cys residues are reduced by ascorbate to free Cys thiols. In this reaction,

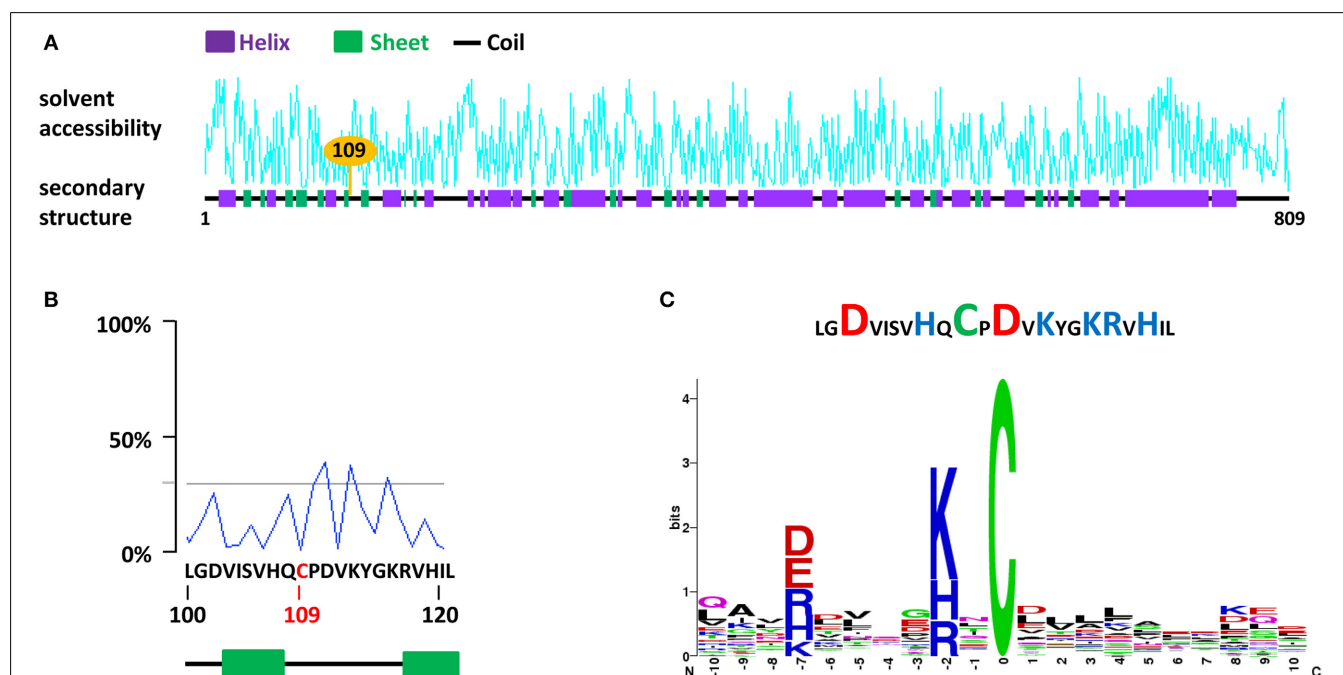


FIGURE 2 | A case study for the search for S-nitrosylation sites in the chaperone protein CDC48 of *A. thaliana* (AtCDC48). (A) Analysis of AtCDC48 using dbSNO 2.0. The Cys-109, found to undergo S-nitrosylation *in vivo* (Fares et al., 2011) and *in vitro* (Astier et al., 2012a) is highlighted. (B)

Details of the solvent accessibility and of the primary and secondary structure surrounding Cys-109. (C) Abundance of basic (blue) and acidic (red) residues flanking Cys-109 (upper panel) and statistically significant conserved motifs surrounding Cys-109 identified using the SNOsite web server (lower panel).

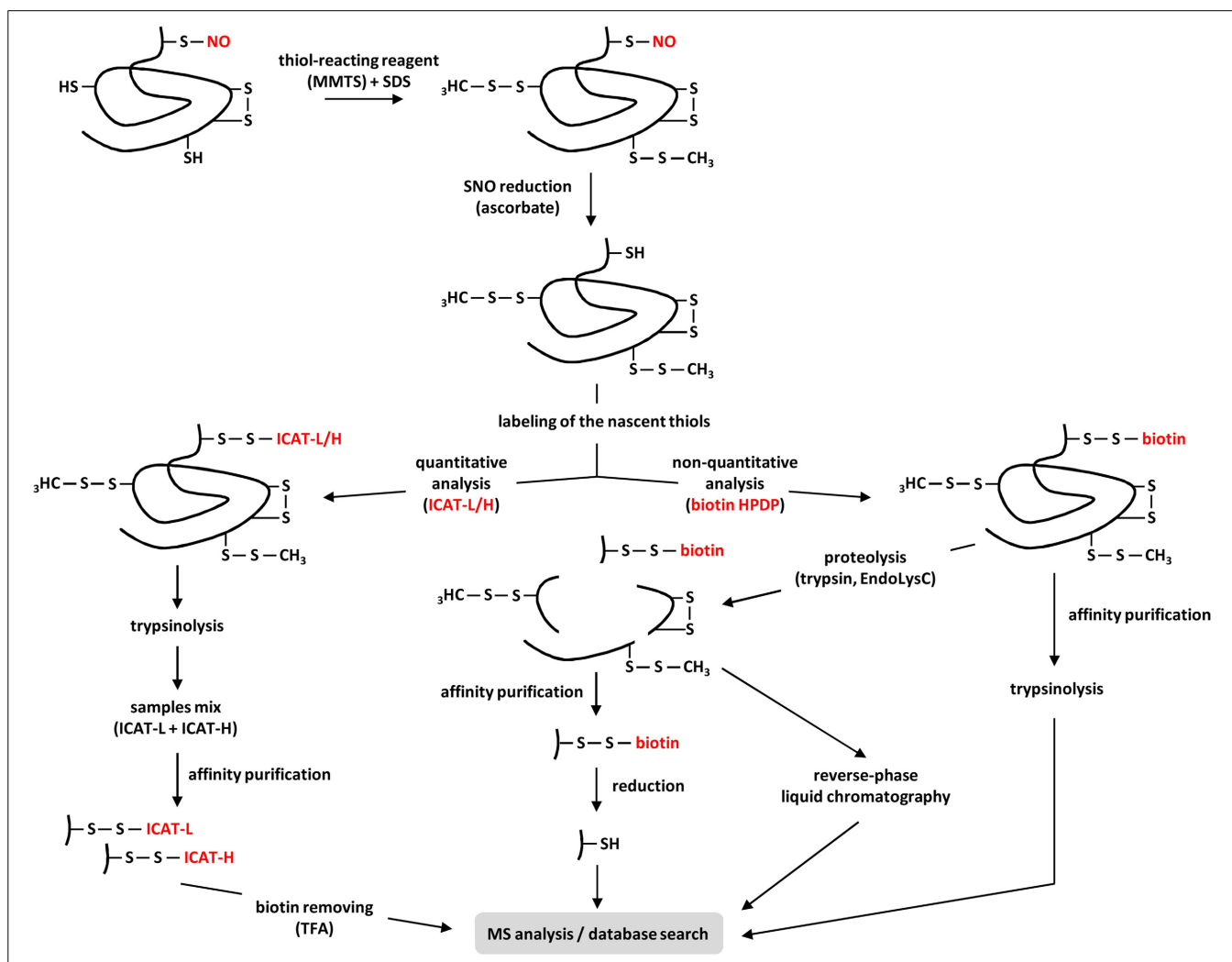


FIGURE 3 | Current proteomic strategies for the identification of

S-nitrosylated proteins in plants. After extraction from plant tissues or cell suspensions, proteins are subjected to the BST. Briefly, after methylthiolation of the free Cys-thiols with methyl-methane thiosulfonate (MMTS) in presence of sodium dodecyl sulfate (SDS), the S-NO bonds of S-nitrosylated proteins are selectively reduced by ascorbate. These steps can also be applied to purified recombinant proteins. Next, the resulting nascent thiols are labeled through two main approaches. In the non-quantitative approach, proteins are marked with a biotin-tagging reagent (usually biotin-HPDP). The resulting biotinylated proteins (e.g., initially S-nitrosylated) are purified by affinity, subjected to trypsinolysis before MS analysis. In order to selectively isolate the peptides undergoing S-nitrosylation, the biotinylated proteins are first

digested by trypsin, Lys-C endoproteinase (EndoLysC) or both before purification by affinity or reverse-phase chromatography. When this latter approach is used, compared to their non-biotinylated counterparts, the biotinylated peptides show a mass shift of +428 Da in MS analysis. When the affinity purification is chosen, the captured biotinylated peptides are eluted by reduction using DTT or 2-mercaptoethanol before MS analysis. In the quantitative approach, nascent thiols are labeled with isotope-coded affinity tags (ICAT) containing ^{12}C (ICAT-L for light) or ^{13}C (ICAT-H for heavy) carbons, depending on the samples. After trypsinolysis, ICAT-L and ICAT-H samples are mixed before avidin purification. Finally, the biotin tags are cleaved by trifluoroacetic acid (TFA) and the ^{12}C - and ^{13}C -labeled peptides are subjected to MS quantitative/qualitative analysis.

ascorbate acts as a nucleophile and undergoes a transnitrosation reaction to yield *O*-nitrosoascorbate which homolyzes into dehydroascorbate and NO (Holmes and Williams, 2000). As reported by Zhang et al. (2005), ascorbate is a poor reducing agent and long incubation times (up to 3 h) as well as high ascorbate concentrations (30 mM or more) significantly improve the sensitivity of the assay. In the third step, the nascent thiols (i.e., initially S-nitrosylated) are biotinylated with a sulfhydryl-specific biotinylating agent, mainly *N*-[6-(biotinamido)hexyl]-3'-(2'-pyridyldithio)propionamide (biotin-HPDP). Importantly, step 2 and 3 occur simultaneously to allow the immediate

biotinylation of the newly liberated thiols. After removing the excess of ascorbate and biotin-HPDP, the resulting biotinylated proteins are enriched through classical purification processes using immobilized avidin or streptavidin and eluted by reduction (using 2-mercaptoethanol or dithiothreitol). Then, the purified proteins are further analyzed by western blotting or mass spectrometry (MS). A proteolytic step carried out with trypsin (Hao et al., 2006) or Lys-C endoproteinase (Astier et al., 2012a) or both (Morisse et al., 2014) can be introduced prior to avidin capture by affinity (Figure 3). The trypsin-based method is known as SNO-Site Identification (SNOSID, Hao et al., 2006). Besides

optimizing the efficiency of the purification step, the main benefit of this strategy is to allow a selective isolation of the biotinylated peptides and therefore a better identification of the S-nitrosylation sites.

Although attractive, the BST is subject to artifacts. Notably, false positives can arise from incomplete blocking of free Cys thiols by the blocking reagent. Furthermore, as highlighted by Forrester et al. (2007), in the presence of indirect sunlight (for instance from a laboratory window), ascorbate reduces biotin-HPDP to biotin-SH, which in turn could lead to artifactual protein biotinylation *via* thiol/disulfide exchange with methylthiolated Cys residues resulting from the first step. Protection from sunlight (e.g., working in darkness) eliminates this risk of false positives. Omission of MMTS, ascorbate and biotin-HPDP are also used as controls. Furthermore, elimination of SNO by photolysis through a strong ultraviolet light source before the application of the BST is recommended as another control for the specificity of the assay (Forrester et al., 2007).

Variants of the BST developed by Jaffrey et al. (2001), including quantitative approaches and the use of protein microarrays have been reported and successfully used (Torta et al., 2008; Astier et al., 2011; Seth and Stamler, 2011; Wang and Xian, 2011; Lee et al., 2014). For instance, Qu et al. (2014) developed a modified BST in which the nascent thiols resulting from ascorbate reduction of the SNO bonds are irreversibly labeled with an isobaric iodoacetyl tandem mass tag (iodoTMT) reagent. The iodoTMT consists of a thiol-reactive iodoacetyl group, a MS-neutral spacer arm and a tandem mass spectrometry (MS/MS) reporter with a mass ranging from 126 (iodoTMT-126) to 131 (iodoTMT-131). When comparing protein samples, it is therefore possible to label each sample with a specific iodoTMT. Then, the differently labeled protein samples are pooled, trypsin-digested and enriched using anti-TMT antibody resin. During MS/MS analysis, the MS/MS reporters are cleaved, thus generating reporter ions with unique m/z (of 126–131). Therefore, this strategy provides an accurate quantification of the S-nitrosylated proteins. Beside such approaches, others have been designed for capturing S-nitrosylated proteins (reviewed by Raju et al., 2012) such as the Trx trapping strategy (Ben-Lulu et al., 2014). This latter method is based on the denitrosylase activity of Trx. The denitrosylation enzymatic process involves the formation of a transient intermolecular disulfide bond between Trx and its S-nitrosylated substrate. Then, thanks to an intramolecular attack mediated by a Trx Cys residue (named the resolving Cys), the denitrosylated target and the oxidized Trx are released. In the Trx trapping strategy, a mutated Trx in which the resolving Cys was mutated is generated in order to stabilize the transient intermolecular disulfide bond between Trx and the S-nitrosylated substrate. In Ben-Lulu et al. (2014) investigation, cell lysates of murine macrophages or human monocytes exposed to lipopolysaccharides/interferon- γ or exogenous NO, respectively, were incubated with the Trx mutant. Subsequently, the proteins captured by Trx were pulled down and released with DTT before identification by MS analysis. Hundreds of S-nitrosylated proteins were next identified including novel candidates playing key functions in cellular homeostasis and signaling.

In plants, identification of S-nitrosylated proteins was based primarily on the initial BST with a few adjustments. Recently,

Fares et al. (2011) and Puyaubert et al. (2014) combined the BST with isotope-coded affinity tags (ICAT) as substitutes for biotin-HPDP to provide a quantitative analysis of proteins constitutively S-nitrosylated or undergoing this PTM in *A. thaliana* cell suspensions facing abiotic stresses. ICAT is based on an iodoacetamide group, which reacts specifically with cysteine thiols, connected to biotin by a linker that contains ^{12}C (light: ICAT-L) or ^{13}C (heavy: ICAT-H) carbons, thus differing in mass by 9 Da. It also contains an acid-cleavable group allowing the removal of the biotin by treating the labeled peptides with trifluoroacetic acid (TFA). In this procedure first reported by Wu et al. (2011), newly exposed free thiols (i.e., initially S-nitrosylated) resulting from ascorbate reduction were differentially labeled with either ICAT-L or ICAT-H (**Figure 3**). More precisely, protein samples extracted from stressed cells were labeled with ICAT-H, while untreated samples resulting from untreated cells were labeled with ICAT-L. After trypsin digestion, ICAT-H- and ICAT-L-labeled peptides were mixed before avidin purification. Finally, the biotin tag was cleaved using TFA in order to generate higher quality MS/MS spectra for the identification and quantification of SNO-peptides.

MASS SPECTROMETRY

Mass spectrometry involves the analysis of ionized molecules with the purpose to determine their structure, molecular weight and abundance. In the biological field, two ionization techniques are mainly used: MALDI and electrospray ionization (ESI). Karas and Hillenkamp (1988) were the first to demonstrate the ability of MALDI to detect molecules in a sub-nanogram range. Fenn et al. (1989) were also able to achieve similar results in the detection of large molecules using ESI. Since the publication of their results, MS has become a powerful analytical tool in a large range of biological investigations. In principle, the workflow of MS consists of ionization of a sample in an ion source, separation of the ionized molecules according to their mass-to-charge ratio in an analyzer, detection of the ionized molecules in a detector and generation of a mass spectrum. Although both MALDI and ESI allow proteins or peptides to be ionized with high sensitivity, important differences exist between both techniques (reviewed in Silva et al., 2013; Chicooree et al., 2014). One of note is related to the insensitivity of MALDI to salt contaminants. This feature makes it a preferred option for analyzing peptides recovered from electrophoresis gels by peptide mass fingerprint (Henzel et al., 1993). Typically, modified protein samples may be separated using SDS-PAGE, digested by a sequence specific protease and analyzed in a mass spectrometer connected to a MALDI or ESI ionization source. The peptides resulting from digestion can be further separated by liquid chromatography in order to facilitate identification. Relying on the mass-to-charge shift of a specific PTM, mass spectrometry can be successfully used to detect and identify the modification. However, for the unequivocal assignment of a given modification site, MS/MS experiments are required to prove that the mass shift detected in the precursor ion is also observed in the fragment ions carrying the modified amino acid residue.

S-nitrosylation sites in plant proteins can be identified by MS using approaches with some differences. For instance, the addition of the NO group molecule to a Cys residue increases its mass to +29 Da. This mass shift can be detected with the proper instrument optimization and sample preparation. However, with

the MALDI source the peptides bearing this mass addition are decomposed upon laser ionization, which makes their identification difficult (Kaneko and Wada, 2003). The ESI source is most commonly used to probe this mass increase and has been addressed in some recent reviews (Foster, 2012; Chen et al., 2013; Devarie-Baez et al., 2013; Chicooree et al., 2014). For instance, peroxiredoxin II E (PrxII E) was shown to undergo S-nitrosylation in *A. thaliana* leaves infected with an avirulent strain of the bacterial pathogen *Pseudomonas syringae* pv. *tomato* (Romero-Puertas et al., 2008). Using ESI-MS/MS, the same team was able to measure the characteristic mass shift of +29 Da of a Cys-containing peptide of PrxII E following its exposure to GSNO (Romero-Puertas et al., 2007). For this purpose, two experiments were performed. In the first one, the recombinant PrxII E protein was treated with GSNO and then processed with trypsin and AspN protease before MS analysis. In the second one, the recombinant protein was first digested and the resulting peptide mixture was treated with the NO donor before MS analysis. As reported by the authors, treating the peptide mixture with GSNO instead of the full-length protein reduced the time between NO donor treatment and MS analysis from about 4 h to 15 min, increasing the yield of detection of the labile SNO modification. On

the other hand, S-nitrosylated Cys residues can also be identified once biotinylated during application of the BST. The resulting peptides can either be separated by reverse-phase liquid chromatography, enriched and analyzed by MALDI-TOF-MS in order to detect the addition of 428 Da (Astier et al., 2012a) or, as discussed previously, captured on immobilized avidin and selectively released from avidin by reduction of the disulfide linker (Hao et al., 2006) (Figure 3). This latter approach presents some drawbacks as labeled peptides are reduced before MS analysis. Consequently, it is not possible, for peptides with multiple Cys residues, to precisely determine the S-nitrosylation site. Besides, non-S-nitrosylated peptides can also be disulfide-bonded to a biotin-labeled peptide, which after reduction may be mistaken as S-nitrosylated. This problem was solved by using acidic conditions to release the biotinylated peptides from resin without the loss of the biotin linker, facilitating thus the identification of the specific site containing the additional mass (Greco et al., 2006). More generally, the number of reports on the application of MS for the identification of S-nitrosylated sites in plant proteins has steadily increased in the last years. With few exceptions, all are based on the BST and differ only in the MS equipment utilized, ion sources and the use of liquid chromatography.

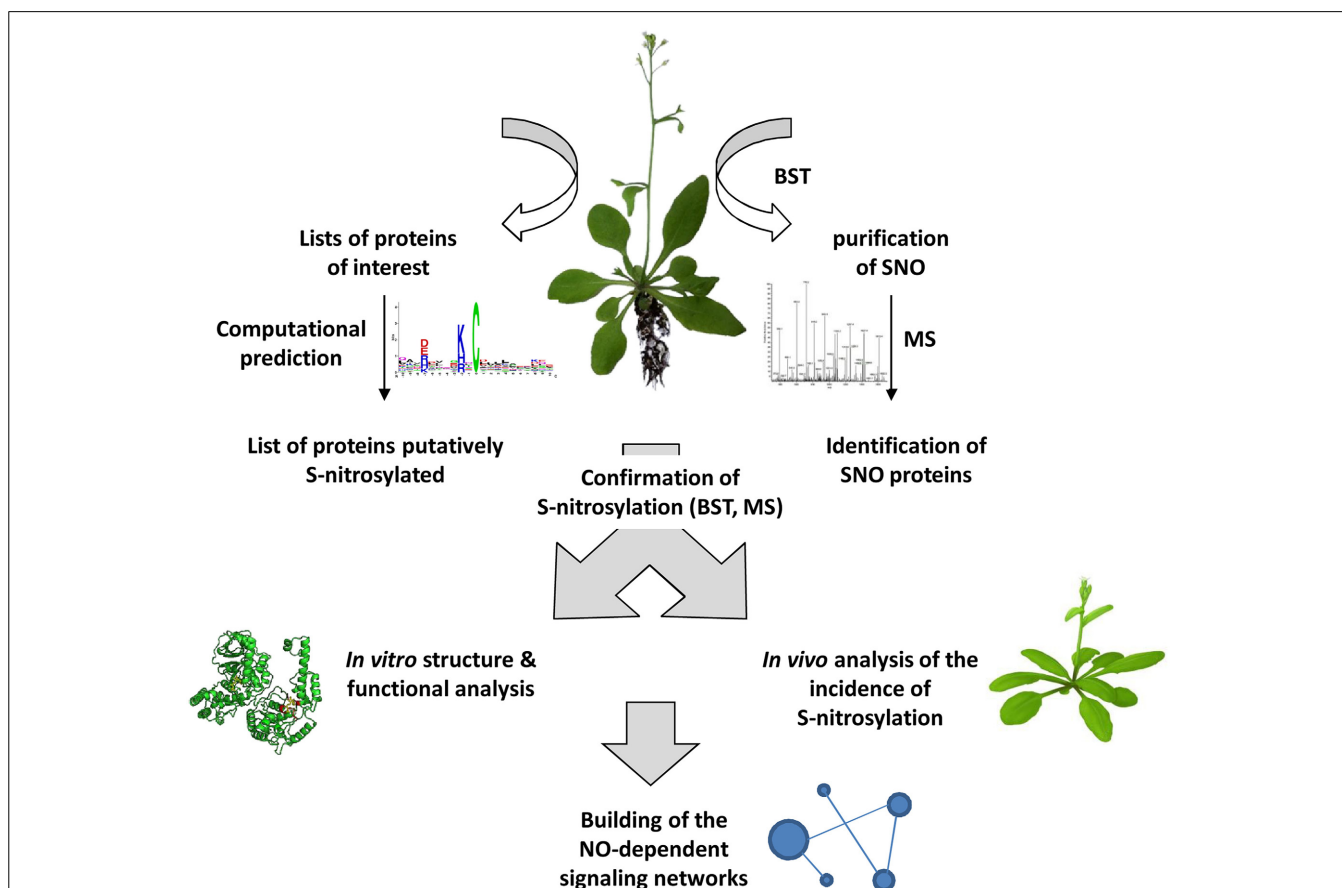


FIGURE 4 | General strategy for investigating S-nitrosylation-based processes in plants. S-nitrosylated proteins (SNO) could be purified and identified from plant tissues using the BST and MS analysis. Alternatively, a search of proteins putatively S-nitrosylated could be undertaken from lists of

proteins involved in particular physiological processes using dedicated web-servers. All the subsequent steps will help in building NO-dependent signaling networks and also will provide new information completing SNO databases and web servers dedicated to SNO sites.

CONCLUSION

The continued success of NO research in plants depends in part on the identification and functional characterization of S-nitrosylated proteins. Such investigation is of importance to advance our understanding of the regulation and organization of the NO-dependent cellular pathways and networks regulating key physiological processes.

During the last few years, there has been an important investment to decipher S-nitroso-proteomes of plant tissues or cell suspensions. BST has been and is still the most commonly used technique for this purpose. Compared to the original protocol described by Jaffrey et al. (2001), few modifications have been made such as a tighter control of ascorbate concentration or the use of thiol-reacting reagents other than MMTS. Undoubtedly, this approach provided significant results and opened new roads of research. Importantly, BST also generates false positives and a thorough analysis of such limitation is still lacking. Furthermore, BST does not allow a precise location of the Cys residue of interest on peptides with multiple Cys residues by MS analysis. Highlighting these drawbacks, for most of the candidates identified so far, a physiological role has not been ascribed to S-nitrosylation. However, at our current level of knowledge, the approach of studying S-nitrosylation by focusing on single proteins is essential and remains the most efficient way to decipher the complexity of the molecular mechanisms inherent to NO physiological function in plants. Therefore, while the list of plant protein candidates for S-nitrosylation is increasing, there is a need to identify the particular Cys residue(s) modified and to define the physiological relevance of their S-nitrosylation. Identification of the Cys residues of interest is also required to further characterize the physico-chemical, biochemical and structural features of S-nitrosylation sites. In addition, this approach will enrich the current databases centralizing S-nitrosylated proteins and, consequently, will help in defining parameters that must be fed into computational prediction of S-nitrosylation sites. Such combinations of proteomic-scale approaches with bioinformatics tools could therefore hold great promise for the elucidation of NO functions in plant cells.

Another issue concerns the quantitative aspects of the BST. So far, few studies provided a quantitative analysis of S-nitrosylated plant proteins. This might be partly explained by the fact that in most of the studies published so far, S-nitrosylation has been investigated through the use of NO donors delivering doses of NO beyond the range of physiological concentrations. Also, such approaches do not take into account the spatial and temporal features of NO-induced PTM. Indeed, it should be emphasized that under physiological contexts, S-nitrosylation is a dynamic and labile PTM restricted to a small subset of proteins which might be located in discrete subcellular compartments. Although useful, NO donors poorly mimic such biological conditions. We assume that quantitative analysis should provide a greater appreciation of this process *in vivo* and a better view of the S-nitrosylation states of proteins, in particular when comparing various conditions. In this regard, the use of isobaric iodoTMT reagents allowing the simultaneous comparison of several samples in a single MS/MS analysis (Qu et al., 2014) looks promising.

In animal biology, the burgeoning of new technologies such as high-density protein microarray chips, and the improvement of

established approaches such as BST and combined MS analysis have contributed to progress in the field of NO research. Plant biologists need to better consider these technological aspects. New tools and techniques are indeed required to provide both quantitative and qualitative data allowing a detailed and integrated insight into S-nitrosylation of plant proteins (**Figure 4**). Accompanying strategies include mutagenesis, structural analysis, subcellular localization, functional genomics and the search of interacting partners. This latter approach is of prime importance as certain S-nitrosylated proteins were shown to belong to protein complexes considered as fundamental building blocks of cellular signaling.

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The effect of soil on cork quality

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The present work aimed to contribute for a better knowledge regarding soil features as cork quality indicators for stoppers. Cork sampling was made in eight Cork oak stands (montados de sobreiro) located in the Plio-Pleistocene sedimentary formations of Península de Setúbal in southern Tagus River region. The samples used to classify the cork as stopper for wine bottles were obtained in eight cork oak stands, covering soils of different types of sandstones of the Plio-plistocene. In each stand, we randomly chose five circular plots with 30 m radius and five trees per plot with same stripping conditions determined by: dendrometric features (HD- height stripping, PBH- perimeter at breaster height), trees vegetative condition (defoliation degree); stand features (density, percentage canopy cover); site conditions (soil type and orientation). In the center of each plot a pit was open to characterize the soil profile and to classify the soil. Cork quality for stoppers was evaluated according to porosity, pores/per cm² and cork boards thickness. The soil was characterized according to morphological soil profile features (lithology, soil profound, and soil horizons) and chemical soil surface horizon features (organic matter, pH, macro, and micronutrients availability). Based on the variables studied and using the numerical taxonomy, we settled relationships between the cork quality and some soil features. The results indicate: (1) high correlation between the cork caliber and boron, cation exchange capacity, total nitrogen, exchange acidity, and exchangeable magnesium, potassium, calcium, and sodium in soils of theirs cork oaks; (2) the cork porosity is correlated with the number of pores/cm² and magnesium soil content; (3) the other soil features have a lower correlation with the caliber, porosity, and the number of pores per cm².

Keywords: technology, forest products, soil, numerical taxonomy, cork quality

INTRODUCTION

Cork is the bark of the cork oak tree which gives rise to cork stoppers used to seal wine bottles. So, the cork is a 100% natural and recyclable product. To produce stoppers with good quality to seal the bottles well, the debarked cork must have specific mechanical features: thickness (caliber) to obtain stoppers with sufficient diameter and low porosity for a good sealing.

In fact cork quality is evaluated differently in the tree, before debarked, or as manufactured stoppers. In the forest, the evaluation is qualitative and subjective, so there is no reproducibility and traceability in its evaluation.

Cork quality is commonly attributed to genetic factors excluding the influence of site conditions (soil and climate). However, it is real that cork features and quality varies widely between different cork-oak regions or even within each region or farm. In any case, it is unknown the influence of the soil features on physical features of the cork. Such knowledge could be very useful to predict cork quality before debarked and its variability on farms.

Thus, given the importance of the cork to the economic sustainability of farms, we think that this article can contribute to a better understanding for the stakeholders regarding the relationships between soil type, soil physical and chemical features, and

physical features of cork. This approach is a tool for predicting cork quality on the tree based on the knowledge of the soil.

MATERIALS AND METHODS

MATERIAL

In this study, 135 trees from 28 circular plots with 30 m radius, distributed by different cork-oak stands at Península de Setúbal, that extends through the region between the Tagus River and the Sado River, were chosen. At least, five trees per plot have been selected for cork samples collection taking in account the uniformity of the soil type. For each plot, the soils were classified according to World Reference Base for soil resources (FAO, 2006).

METHODS

Cork sample preparation

Cork samples were obtained by direct extraction from the trees. One sample with 20 × 20 cm square was obtained from each selected tree. Then, the cork samples were boiled for 1 h in order to improve its mechanical capabilities (thickness and porosity), placed in a ventilated area and pressed in order to straighten the corks until to be boards, so that subsequent procedures were more workable.

Cork thickness

The thickness of cork boards was measured with a caliper to an accuracy of 0.02 mm. The measurements were performed at both ends of the boards. The thickness was obtained by the average of the two determinations.

Cork porosity

The study of cork porosity was made through image analysis with a digital camera with 6 mega Pixels (Pestana, 2003). The samples were sanded to rectify the surface and subjected to a jet of compressed air to clean the surface, so that they can get an image with clear and defined pores.

In addition, it was also determined the number of pores/cm². These two parameters give a good knowledge regarding cork quality.

Soils classification

The soil features and classification were obtained from the observation of the soil profile in each plot. To do this, we performed the holes or pits opening to describe the morphological features along the profile and to obtain samples for physical and chemical analysis. The physical and chemical soils analysis was performed according to the analytical methods at Laboratory Rebelo da Silva (LQARS).

The soil of each plot it was classified according to FAO legend (WRB for soil resources, 2006). According to this classification, taking into account the profiles morphological characteristics and the analytical results of the soil, three dominant soil types were identified among the 27 plots: 8 plots are located in Dystric Cambisols, 10 plots in Albic Arenosols, and 6 plots in Plinthic Podzols.

Soils analysis

For the present study the following morphological and physical parameters of soil profile were considered: presence of ground-water horizons, depth to which the presence of cork oak roots was observed, thickness of surface horizon and total thickness of the surface and sub-surface horizons. Regarding the chemical parameters only the values observed in the surface horizon of the soil were considered for the following parameters: organic matter content, total nitrogen and organic carbon, assimilable phosphorus, and potassium (P₂O₅, K₂O), micronutrients (Mn, and B), pH, exchange acidity, and the exchangeable bases (Ca²⁺, Mg²⁺, K⁺, and Na⁺).

The analytical methods used for soil features characterization were:

Organic matter it was calculated multiplying the organic carbon content calculated by the Tinsley method by the factor 1.724. Results were expressed in weight of C per kg of soil.

Nitrogen was determined by catalytic pyrolysis using the auto-analyzer NSC2000. Results are expressed in weight of N per kg of soil of dry matter.

The pH was determined by potentiometry in water using a suspension 1:2.5 of the soil sample.

The extractable phosphorus and potassium were determined by the method of Egner-Riehm modified using an extractant

solution of ammonium lactate and acetic acid at pH 3.7–3.8. The phosphorus was quantified by colorimetry according vanamolibdato ammonium method and potassium by flame photometry. Results are expressed mg/kg of P and K per kg of soil (mgkg⁻¹).

The extractable Magnesium it was determined ammonium acetate at pH 7 method. The results are expressed in mg of Mg per kg of soil.

Exchangeable bases (Ca⁺⁺, Mg⁺⁺, K⁺, and Na⁺) and exchangeable acidity (H⁺ + Al³⁺) were determined by the Mehlich method (1958) using an extractant solution of barium-triethanolamine chloride adjusted to pH 8.1. Ca and Mg were assayed by atomic absorption spectrophotometry with addition of lanthanum chloride and K and Na by flame photometric. The results are expressed in mg per kg of soil cmol (+). Kg⁻¹, and the saturation degree of saturation expressed as a percentage.

Extractable micronutrients (Fe, and B): Fe was determined by Lakanen e Ervio Method (1971) using an extractable solution of ammonium nitrate (5 M) with acetic acid (0.5 M) and EDTA sodic salt (0.02 M) adjusted at pH 4.65. Results were obtained by absorption spectrophotometry and expressed in mg of each micronutrient per kg of soil; Boron was extracted in boiling water for 5 min and titrated by Curcumin-oxalic acid method (LQARS, 1988). The results are expressed in mg of B per kg of soil.

DATA STATISTICAL ANALYSIS

The plots characterization was supported by the average values of physical parameters of cork. This procedure is due to the fact that

Table 1 | Correspondence between the variables and the codes adopted.

Features	Units	Code
Caliber	(mm)	Caliber
Porosity	(%)	Poro
Number of pores per cm ²		Npor
Surface horizon thickness	(cm)	EspA
Total surface and subsurface horizons thickness	(cm)	EspA+B
Organic matter content	(gkg ⁻¹)	MO
Exchangeable calcium	(cmol _(c) kg ⁻¹)	Ca ⁺⁺
pH		pH
Exchangeable magnesium	(cmol _(c) kg ⁻¹)	Mg ⁺⁺
Exchangeable potassium	(cmol _(c) kg ⁻¹)	K ⁺
Exchangeable sodium	(cmol _(c) kg ⁻¹)	Na ⁺
Cation exchange capacity	(cmol _(c) kg ⁻¹)	CTC
Base saturation degree	(cmol _(c) kg ⁻¹)	GSB
Exchange acidity	(cmol _(c) kg ⁻¹)	AT
Exchangeable bases sum	(cmol _(c) kg ⁻¹)	S
Total nitrogen	(gkg ⁻¹)	Ntot
Extratable phosphorus	(mgkg ⁻¹)	P2O5
Extratable potassium	(mgkg ⁻¹)	K2O
Extratable magnesium	(mgkg ⁻¹)	Mg
Boron	(mgkg ⁻¹)	B

it is impossible to accurately determine the soil parameters for each tree, thus obtaining the mean values of these cork features. In Table 1, we have adopted codes for the features studied.

Numerical taxonomy analysis

For each data sets was prepared (in advance) a data matrix where rows correspond to the plots Operational Taxonomic Units (OTUs), and the columns correspond to the variables determined (Pestana, 2003).

How there is a different nature of the various variables, we proceeded to the standardization of the original matrix. Then, we obtain a new matrix with standardized data, where the average value of each feature is now zero and its variance 1. In this operation, it is calculated the mean, and standard deviation for each feature and then it replaces each original value by dividing their difference to the mean and its standard deviation (Carneiro, 1987; Pestana, 2003).

We calculated the similarity coefficient using the average Euclidean distance. This coefficient represents the distance between the representative points of two samples in space, which will have as many dimensions as the number of features used. In

case of equal analyzing objects, this distance is zero and increases with the dissimilarity between them (Carneiro, 1987; Pestana, 2003).

Of the various methods of aggregation of sequential, agglomerative, hierarchical and non-override type, i.e., the type SAHN, we used the Unweighted Pair—Group Method Using Arithmetic Averages (UPGMA) (Sneath and Sokal, 1973; Pestana, 2003).

The results thus obtained are presented on the form of a branched structure, in which the different branches are related according to the values of the similarity measures which are based on the agglomeration method, which is known as phenogram (Pestana, 2003).

For this phenogram, we calculated the cophenetic correlation coefficient (Sokal and Rohlf, 1962) between the matrix of cophenetic values, expressing the relationship of similarity between the implicit OTUs in phenogram, and similarity matrix (or dissimilarity) was calculated. The cophenetic correlation coefficient indicates the degree of agreement between the two matrixes, allowing assess whether the phenogram is an acceptable representation of those distances (Carneiro, 1987; Pestana, 2003).

Table 2 | Cork quality and physical and chemical soil data.

Plots	Cork quality data			Soil physical data			Soil chemical data														
	Poro	Npor	Caliber	EspA	EspA+B	MO	Ntot	pH	P ₂ O ₅	K ₂ O	Mg	B	Ca ⁺⁺	Mg ⁺⁺	K ⁺	Na ⁺	AT	S	CTC	GSB	
CLMPS/2	18.91	0.06	28.63	15.0	40.0	1.78	0.06	5.7	53.0	49.0	25.0	0.05	1.46	0.25	0.09	0.11	1.50	1.92	3.4	56.2	
CLMPS/3	20.07	0.06	34.93	17.5	32.0	1.05	0.03	5.8	12.0	29.0	13.0	0.05	0.77	0.15	0.06	0.02	1.00	0.99	2.0	49.9	
CLMPS/4	38.80	0.07	40.53	15.0	22.5	0.23	0.01	5.7	3.0	37.0	9.0	0.05	0.30	0.07	0.07	0.03	1.00	0.46	1.5	31.7	
CLMPS/5	32.33	0.08	33.76	15.0	35.0	1.91	0.06	5.6	33.0	124.0	18.0	0.05	1.35	0.17	0.15	0.02	1.50	1.70	3.2	53.1	
CLMM/2	12.87	0.05	30.91	15.0	40.0	1.34	0.04	5.7	12.0	47.0	19.0	0.05	1.26	0.17	0.09	0.02	1.40	1.54	2.9	52.3	
CLMM/3	19.26	0.07	31.02	12.5	32.5	1.24	0.04	6.1	17.0	49.0	23.0	0.05	0.96	0.21	0.10	0.04	0.70	1.31	2.0	65.2	
CLMM/4	30.66	0.05	29.19	20.0	42.5	0.91	0.03	5.8	9.0	25.0	14.0	0.05	0.81	0.15	0.05	0.12	1.00	1.12	2.1	52.8	
CLMM/5	31.24	0.06	32.01	12.5	30.0	1.08	0.03	5.6	6.0	32.0	12.0	0.05	0.85	0.13	0.06	0.12	1.90	1.16	3.1	38.0	
HEMO5	3.97	3.68	37.69	22.5	60.0	0.88	0.03	8.3	69.0	40.0	44.0	0.05	4.47	0.42	0.05	0.03	0.00	4.97	5.0	100.0	
HEMO6	3.97	3.07	34.01	30.0	72.5	0.86	0.03	5.4	4.0	151.0	232.0	0.58	1.50	2.27	0.37	0.08	2.00	4.22	6.2	67.8	
HEMR14	5.20	3.83	35.67	27.5	47.5	0.40	0.01	5.4	9.0	39.0	19.0	0.05	1.25	0.17	0.08	0.01	1.70	1.51	3.2	47.0	
HEMR14a	5.30	5.12	31.51	20.0	35.0	2.13	0.06	6.5	17.0	101.0	30.0	0.33	1.93	0.32	0.22	0.03	1.00	2.49	3.5	71.4	
HEPMR42	13.21	7.18	36.25	25.0	47.5	1.24	0.03	7.4	28.0	68.0	42.0	0.05	2.51	0.29	0.11	0.02	0.00	2.94	2.9	100.0	
HEPMR42a	4.21	4.71	25.45	20.0	50.0	1.27	0.04	7.2	27.0	48.0	43.0	0.25	2.55	0.44	0.09	0.02	0.00	3.10	3.1	100.0	
HEPMR44	4.04	3.29	31.94	20.0	47.5	0.92	0.02	6.2	9.0	39.0	25.0	0.05	0.89	0.23	0.08	0.01	1.10	1.21	2.3	52.4	
HEPMR44a	8.23	5.82	36.45	20.0	45.0	1.38	0.04	7.4	25.0	36.0	13.0	0.05	2.35	0.28	0.06	0.02	0.00	2.71	2.7	100.0	
HEPMR52a	3.71	0.03	29.29	30.0	60.0	1.09	0.02	6.2	7.0	36.0	11.0	0.05	0.83	0.24	0.08	0.02	1.20	1.17	2.4	49.3	
VCVC1	8.99	5.42	34.46	15.0	45.0	1.31	0.04	5.7	15.0	95.0	18.0	0.05	0.68	0.19	0.26	0.01	1.90	1.13	3.0	37.4	
VCVC2	6.54	4.99	40.67	22.5	27.5	0.80	0.02	5.3	13.0	21.0	13.0	0.05	0.48	0.11	0.05	0.01	1.50	0.64	2.1	30.0	
VCVC3	6.71	3.85	42.92	17.0	21.0	0.98	0.03	5.3	14.0	41.0	46.0	0.05	0.80	0.17	0.07	0.03	1.60	1.06	2.7	39.9	
VCVC4	8.17	4.61	50.52	17.5	35.0	2.54	0.07	5.9	12.0	119.0	90.0	0.05	2.00	0.80	0.23	0.04	1.70	3.07	4.8	64.4	
VCVC5	7.61	4.61	46.96	20.0	35.0	1.90	0.06	6.0	15.0	102.0	70.0	0.26	1.59	0.43	0.21	0.02	1.30	2.25	3.5	63.4	
VC1	4.01	3.61	32.32	30.0	45.0	1.60	0.04	5.7	5.0	35.0	40.0	0.19	0.98	0.25	0.10	0.02	1.60	1.35	3.0	45.7	
VC2	2.48	3.53	30.26	25.0	65.0	1.30	0.03	5.2	8.0	24.0	36.0	0.19	0.78	0.20	0.05	0.01	1.70	1.05	2.8	38.2	
VC3	5.69	3.71	26.17	25.0	80.0	0.08	0.03	5.5	5.0	24.0	40.0	0.19	0.60	0.19	0.05	0.01	1.30	0.85	2.2	39.6	
VC4	3.87	3.10	31.00	30.0	50.0	1.20	0.04	5.4	7.0	31.0	24.0	0.19	0.71	0.19	0.06	0.02	1.90	0.98	2.9	33.9	
VC5	5.26	3.86	29.25	25.0	70.0	1.55	0.05	5.4	10.0	41.0	44.0	0.19	0.96	0.30	0.09	0.01	1.30	1.36	2.7	51.0	
CI	2.84	1.90	21.00	25.00	65.00	0.85	0.03	5.4	5.0	20.0	24.0	0.19	0.35	0.12	0.05	0.01	1.90	0.53	2.4	21.7	

It used also another method of aggregation for better understanding of the results the aggregation method called Minimum Spanning Tree (MST). To obtain the graphical representation of the studying objects along axes, a small number of dimensions, usually two or three, retaining as much of the variability of the original multidimensional matrix data, we selected the ordination method on main components. The projections of the variables that characterize the objects studied in the first two main components, which allows us to analyze the contribution of each of the spatial arrangement of the objects studied were made (Reis, 2001).

RESULTS AND DISCUSSION

To perform this analysis, we used a data matrix with 28 plots (rows) and the 20 physical features of corks (including image analysis) and physical and chemical soil features (columns, Table 2).

The phenogram (Figure 1) obtained from the distance matrix (using the UPGMA method) is suitable for the respective matrix because of the cophenetic coefficient correlation ($r = 0.72$). Observing the phenogram it is possible to verify the establishment of two groups. One made by plots CLMPS/5, VCVC1,

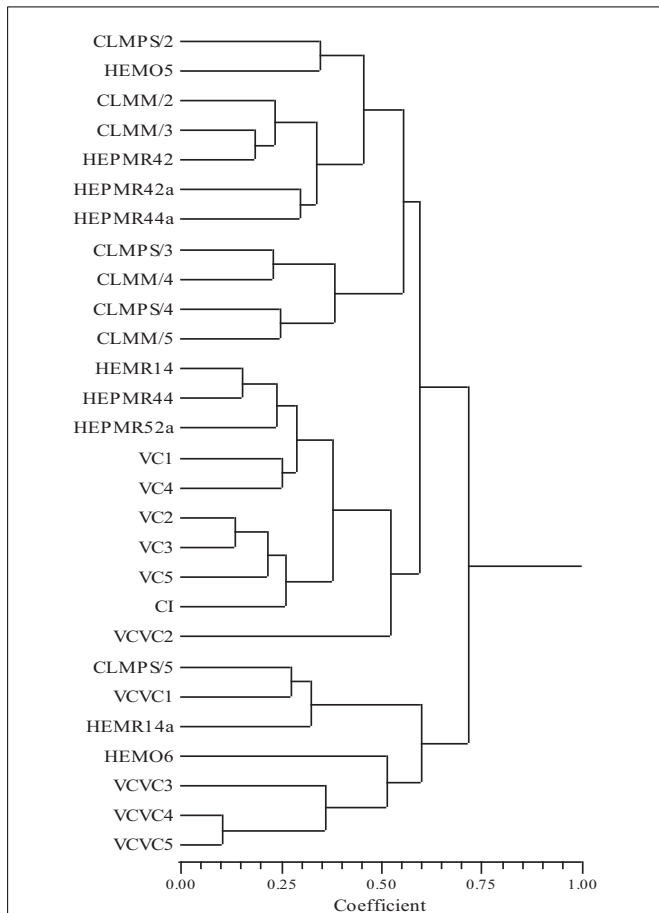


FIGURE 1 | Phenogram of 28 plots, based on the UPGMA method applied to the distances matrix ($r = 0.72$).

HEMR14a, HEMO6, VCVC3, VCVC4, and VCVC5 and another with the remaining plots. This last group, we might consider subdivide into two groups composed by: the first includes the CLMPS/2, HEMO5, CLMM2, CLMM3, HEPMR42, HEPMR42a, HEPMR44a, CLMPS/3, CLMM4, CLMPS4, and CLMM/5; the second group is composed by the remaining plots.

The projection of the 28 plots in the spatial defined by the three main axes, which together account for 77.71% of total variance, which was overlaid with MST (Figure 2), allows to confirm the groupings obtained by the phenogram. The analysis of Figure 2

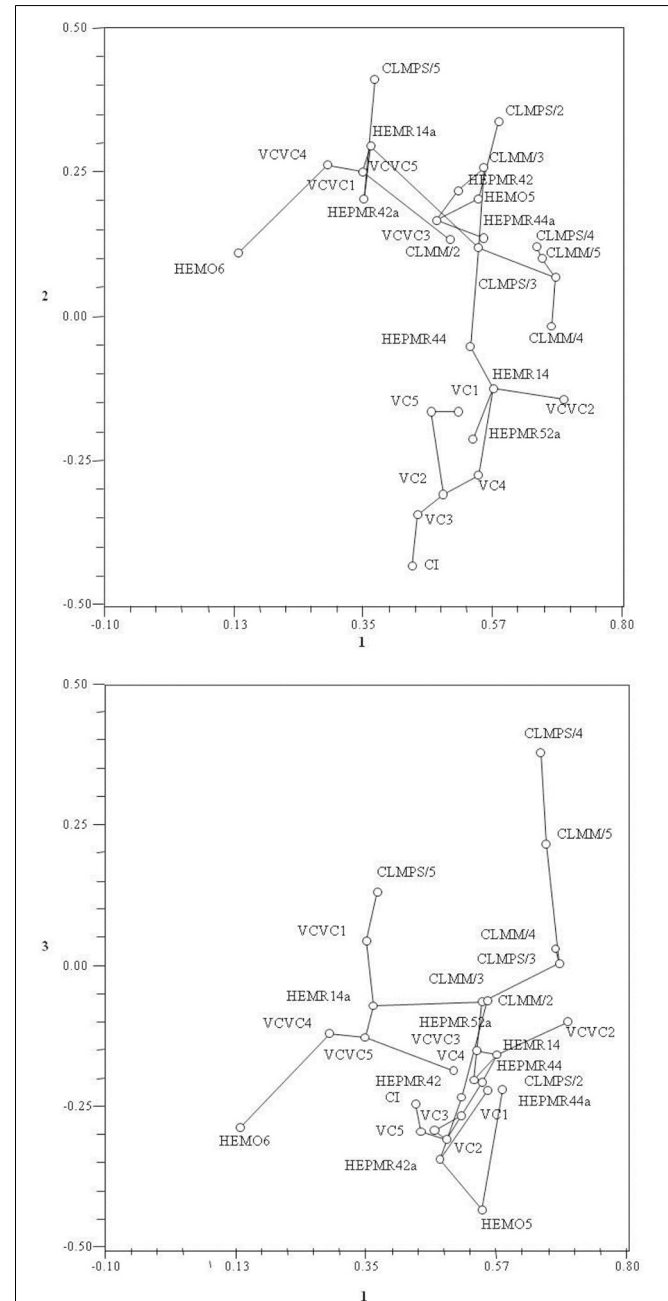
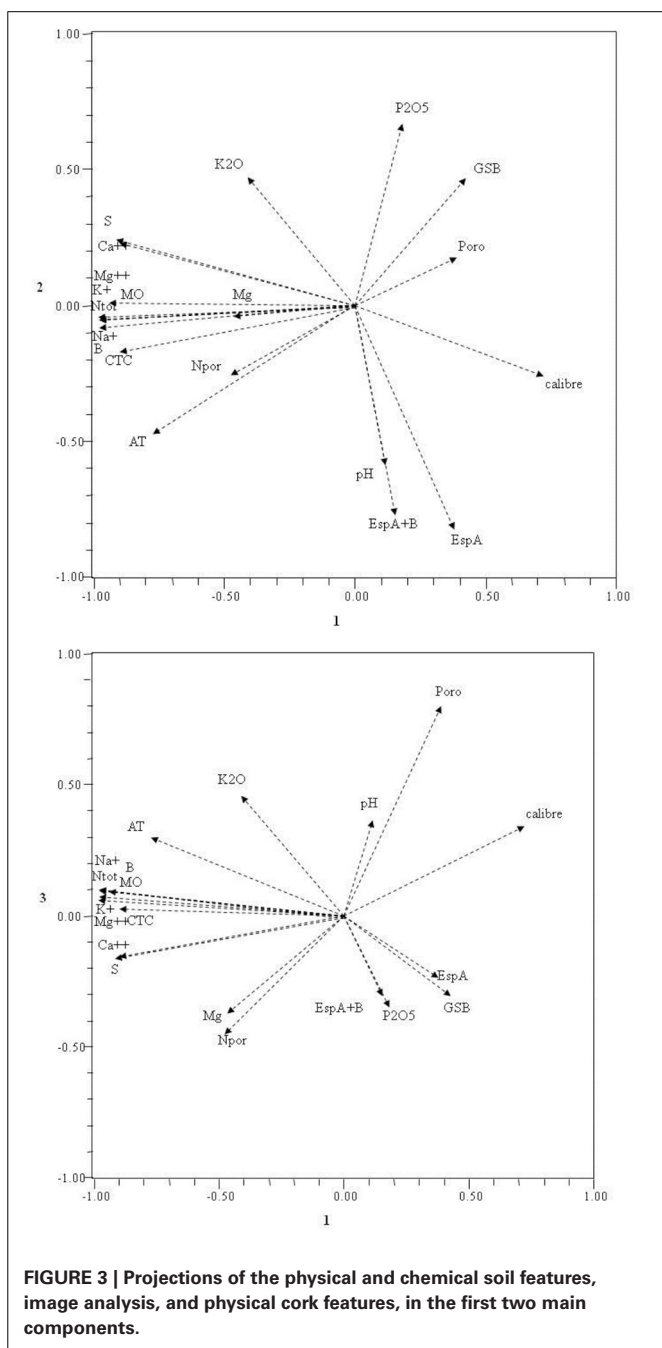


FIGURE 2 | Projections of the 28 plots in the plane defined by the two first main axes, which was overlaying the Minimum Spanning Tree.



allows us to say that the links and the spatial arrangement of the plots are in agreement with most clusters determined in the phenogram.

Figure 3 shows the contributions of variables to the spatial distribution of the plots.

The first main component is controlled by organic matter (MO), total nitrogen (Ntot), boron (B), exchangeable calcium (Ca^{++}), magnesium (Mg^{++}), potassium (K^{+}), and sodium (Na^{+}), exchangeable bases sum (S), cation exchange capacity (CTC), exchange acidity (AT), and caliber (Caliber). The second component is controlled by the most important variables for

pH (pH), surface horizon thickness (EspA), total surface and subsurface horizons thickness (EspA+B), phosphates (P_2O_5), potassium oxide (K_2O), and sum of basic cations (GSB). The third is controlled by the magnesium (Mg), porosity (Pore), and pores number/ cm^2 (Npor).

Analyzing the two **Figures 2, 3**, we find that the first main component separates to the right side, the plots with differences in cork caliber, and to the left, the soil features in total nitrogen, cation exchange capacity, organic matter, boron, sulfur, and exchangeable sodium, magnesium, calcium, and potassium.

The second main component drives to the down region of the same figures (**Figures 2, 3**), plots with high values of surface horizon thickness, total surface and sub-surface horizons thickness and pH, and to upper region the potassium oxide, phosphates, and sum of basic cations. The third main component drives the plots with corks high porosity to the upper side of the plane (1,2) and the magnesium and number of pores/ cm^2 to the opposite side of the same plane.

We see also a high correlation between the cation exchange capacity and the exchangeable calcium, organic matter, total nitrogen, exchangeable magnesium, potassium, and sodium that indicates that these features are mutually dependents.

We found that the plots of corks with high porosity and low number of pores/ cm^2 are from corks on soils containing lower levels of magnesium. Lower values of boron, cation exchange capacity, total nitrogen, exchange acidity, and exchangeable magnesium, potassium, calcium, and sodium are in plots where de corks have a high caliber.

CONCLUSIONS

Regarding to the foregoing, we conclude that there is a high correlation between the cork caliber and boron, cation exchange capacity, total nitrogen, the exchange acidity, and exchangeable magnesium, potassium, calcium, and sodium in soils of their cork oaks, i.e., lower values of boron, cation exchange capacity, total nitrogen, the exchange acidity, and exchangeable magnesium, potassium, calcium, and sodium are in plots where de corks have a high caliber. It was also found that the cork porosity is correlated with the number of pores/ cm^2 and magnesium, i.e., the number of pores/ cm^2 and magnesium varies inversely with the cork porosity.

The other soil features have a lower correlation with the caliber, porosity, and the number of pores per cm^2 .

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Down-regulation of *Fusarium oxysporum* endogenous genes by Host-Delivered RNA interference enhances disease resistance

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Fusarium oxysporum is a devastating pathogen causing extensive yield losses in a variety of crops and development of sustainable, environmentally friendly methods to improve crop resistance is crucial. We have used Host-Delivered RNA interference (HD-RNAi) technology to partially silence three different genes (*FOW2*, *FRP1*, and *OPR*) in the hemi-biotrophic fungus *F. oxysporum* f. sp. *conglutinans*. Expression of double stranded RNA (dsRNA) molecules targeting fungal pathogen genes was achieved in a number of transgenic Arabidopsis lines. *F. oxysporum* infecting the transgenic lines displayed substantially reduced mRNA levels on all three targeted genes, with an average of 75, 83, and 72% reduction for *FOW2*, *FRP1*, and *OPR*, respectively. The silencing of pathogen genes had a clear positive effect on the ability of the transgenic lines to fight infection. All transgenic lines displayed enhanced resistance to *F. oxysporum* with delayed disease symptom development, especially *FRP1* and *OPR* lines. Survival rates after fungal infection were higher in the transgenic lines compared to control wild type plants which consistently showed survival rates of 10%, with *FOW2* lines showing 25% survival; *FRP1* lines 30–50% survival and *OPR* between 45 and 70% survival. The down-regulation effect was specific for the targeted genes without unintended effects in related genes. In addition to producing resistant crops, HD-RNAi can provide a useful tool to rapidly screen candidate fungal pathogenicity genes without the need to produce fungal knockout mutants.

Keywords: Host-Delivered RNAi, host-induced gene silencing, *Fusarium oxysporum*, disease resistance, disease control, plant fungal pathogens

INTRODUCTION

The genus *Fusarium* includes several species of fungi that are broadly spread in soil and organic substrates worldwide. *Fusarium oxysporum* is one of the most relevant species of this genus and is the causal agent of root rots, damping-off and wilt diseases in more than 100 plants species, including a wide range of economically important horticultural crops, flowers, trees, and a number of field crops such as cabbage, banana, and cotton (Michielse and Rep, 2009; Takken and Rep, 2010; Dean et al., 2012; Swarupa et al., 2014). *F. oxysporum* consists of over 120 *forma specialis* (f. sp.) of pathogenic strains determined by their primary host plants (Armstrong and Armstrong, 1981; Di Pietro et al., 2003; Fourie et al., 2011). All strains of *F. oxysporum* are saprophytic, being able to grow and survive for long periods on organic matter in soil making it very difficult to control (Olivain and Alabouvette, 1997). Its pathogenic life cycle starts with spore germination upon recognition of a suitable host; once the hyphae is formed, the pathogen enters its host by directly penetrating the roots and colonizes it within the xylem by producing microconidia which leads to mycelia formation (Di Pietro et al., 2003). Colonization and toxin production by the pathogen

results in blockage of the host vascular system (Michielse and Rep, 2009), causing characteristic disease symptoms including vascular yellowing, vein clearing, chlorosis, and necrosis in leaf veins and leaves, leaf detachment and wilting (Di Pietro et al., 2003; Czymmek et al., 2007). After the plant dies, the fungus sporulates on the decayed leaf surfaces. *F. oxysporum* is most prevalent in tropical and subtropical regions and it is expected that its geographical range will extend due to climate change (Okubara and Paulitz, 2005).

Current control methods for *Fusarium* wilt are very limited with crop rotations being ineffective due to the large host range and its persistence in soil (Davis et al., 2006). Management of *Fusarium* wilt is mainly done through cultural practices and farm hygiene which only reduce the transmission of inoculum while soil sterilization can only be performed in glasshouses (Michielse and Rep, 2009). Soil fumigation using broad-spectrum biocides such as methyl bromide is expensive and has many hazardous effects on the environment (Fravel et al., 2003; Davis et al., 2006). Natural resistance on a gene for gene relationship between hosts and *F. oxysporum* races has been described and used to develop resistant crop varieties providing an environmentally safe control

method (Roncero et al., 2003; Michiels and Rep, 2009). However, breeding for resistance is not always an easy process and new races of the pathogen can develop to overcome host resistance (Fravel et al., 2003).

RNA interference (RNAi) is a cellular process found in most eukaryotes involved in developmental regulation of gene expression as well as defense against viruses and transposons (Fire et al., 1998; Watson et al., 2005; Mahmood-Ur-Rahman et al., 2008; Jinek and Doudna, 2009; Obbard et al., 2009). RNAi is triggered by the presence of double stranded RNA (dsRNA) in the cell and ultimately leads to the degradation of homologous single stranded RNA molecules, inhibition of translation and modification of homologous genomic sequences (Qi and Hannon, 2005; Mahmood-Ur-Rahman et al., 2008). The artificial manipulation of this pathway has allowed the efficient silencing of genes in transgenic plants and provided a powerful platform for reverse genetics studies (Duan et al., 2012). An alternative approach involving RNAi, known as Host-Delivered RNAi (HD-RNAi) or Host Induced Gene Silencing (HIGS), has emerged in recent years that use the host plant as a delivery system to cause gene silencing in the pathogen (Fairbairn et al., 2007; Mahmood-Ur-Rahman et al., 2008). In the HD-RNAi approach, the host plant is transformed with a hairpin construct targeting a pathogen gene. Once the transgenic plant is infected and the pathogen starts feeding from the host, the small interfering RNA (siRNA) molecules are transferred to the pathogen cells, ultimately inducing an RNAi response in the pathogen and the silencing of the targeted gene.

HD-RNAi have been proven to work on many plant pathogens such as root knot nematodes where the silencing observed in nematodes feeding from transgenic plants expressing RNAi constructs was specific for the targeted genes with no off-target effects and, in some cases, the transgenic plants were resistant to nematode colonization (Huang et al., 2006; Fairbairn et al., 2007; Dubreuil et al., 2009). RNAi has been used to confer resistance against bacterial pathogens, including crown gall disease caused by *Agrobacterium tumefaciens* (Escobar et al., 2001, 2002; Escobar and Dandekar, 2003; Viss et al., 2003). HD-RNAi is also effective in plant-insect systems and has been used to effectively silence genes in cotton bollworm larvae (*Helicoverpa armigera*) and down-regulation of the *V-ATPase A* gene in western corn rootworm (*Diabrotica virgifera virgifera*) larvae resulted in reduced damage to cotton plants (Baum et al., 2007; Mao et al., 2007). HD-RNAi has been successfully used to silence genes in parasite weeds providing partial protection to the host plant (Tomilov et al., 2008; Aly et al., 2009). Curiously, the RNAi effect can go both ways, as RNAi signals have also been proven to travel from the parasitic plant to the host (Tomilov et al., 2008). On the other hand, HD-RNAi has been proven ineffective in the control of *Striga asiatica*, an economically important parasite of maize (de Framond et al., 2007). The application of HD-RNAi to fungal pathogens is quite recent and has used a number of different approaches to produce dsRNA molecules in the host plant. Mixed success to provide disease resistance against the rust fungi *Puccinia striiformis* and *Puccinia triticina* using the virus-induced gene silencing (VIGS) technique has been reported (Yin et al., 2011; Panwar et al., 2013a,b). Particle bombardment of hairpin constructs to barley and VIGS in wheat resulted in

down-regulation of genes in the powdery mildew *Blumeria graminis* (Nowara et al., 2010). Efforts to use HD-RNAi in oomycetes have been so far unsuccessful (Zhang et al., 2011). Furthermore, a recent report has measured the *in vitro* antifungal activities of a set of synthetic dsRNAs applied to fungal spores of *F. oxysporum* f. sp. *cubense* and *Mycosphaerella fijiensis*, the causing agents of Fusarium wilt and black sigatoka in bananas, respectively (Mumbanza et al., 2013). The results are promising and indicate that some of the synthetic dsRNAs resulted in inhibition of spore germination. HD-RNAi has been recently used on several *Fusarium* species. *GUS* gene expression in *F. verticillioides* was significantly suppressed after feeding on transgenic tobacco plants expressing *GUS*-RNAi (Tinoco et al., 2010). More recently, HD-RNAi approach was also tested in Arabidopsis and barley against *F. graminearum*. It was demonstrated that HD-RNAi effectively silenced three fungal cytochrome P450 lanosterol C-14 α -demethylase (CYP51) genes and resulted in strongly enhanced resistance to the pathogen (Koch et al., 2013).

F. oxysporum (f. sp. *conglutinans*) infects the family *Brassicaceae* including Arabidopsis. It is known that in Arabidopsis, *F. oxysporum* enters the host through the primary root tip and lateral root emerging points (Di Pietro et al., 2001; Czymbek et al., 2007). Normally 3–4-week-old Arabidopsis plants develop characteristic chlorosis along the leaf veins 5–7 days after inoculation (Trusov et al., 2013). The susceptible genotypes decay approximately 20 days after inoculation, and distinguished fungal sporulation occurs on the plant surfaces. There are obvious advantages in using this particular host-pathogen interaction system. Firstly, simple inoculation assays that resemble natural infection process starting from roots progressing to vasculature have been developed (Trusov et al., 2013). Secondly, this system allows quantification of disease symptoms, as the number of leaves showing chlorotic veins per plant can be recorded throughout the plant disease progression (Trusov et al., 2013). Finally, aside from symptom quantification, the survival percentage by the number of plants which has thrived 15 days after inoculation can also be counted (Trusov et al., 2013). Here, we have produced transgenic Arabidopsis lines expressing hairpin constructs targeting three different *F. oxysporum* genes and shown down-regulation of all targeted genes in fungus infecting the transgenic lines. The effect of the silencing on disease progression and ultimate survival of the plants is evaluated and effectiveness of HD-RNAi technology in practice is discussed.

MATERIALS AND METHODS

PATHOGEN PREPARATION AND INOCULATIONS

F. oxysporum (f. sp. *conglutinans*) (BRIP 5176; Department of Primary Industries, Queensland, Australia) were grown and plants were inoculated as previously described (Trusov et al., 2006, 2013). In summary, plants were dipped into the fungal inoculum for 30 s after carefully cleaning the roots with water. *Fusarium* inoculated plants were replanted into fresh soil and grown at 28°C in a growth cabinet with required humidity and light intensity. Twenty plants from each transgenic line as well as wild type were inoculated. The degree of infection was measured as symptoms appeared and progressed in a time span of 7–12 days

post inoculation (dpi), by counting the number of leaves with yellow veins. The total number of leaves was also counted and found to be the same in all genotypes. The percentage of surviving plants out of 20 plants was recorded after a minimum of 15 dpi. Three infection experiments were performed for each gene with similar results. Fungal RNA was extracted from the aerial part of Arabidopsis plants 7 days after inoculation as described previously (Chakravorty et al., 2011).

CONSTRUCTION OF PLASMID AND PLANT TRANSFORMATION

The *F-box protein Required for Pathogenicity 1* (*FRP1*) (Genbank AY673970.1), *F. oxysporum* Wilt 2 (*FOW2*) (GenBank AB266616.1) and a previously uncharacterized gene with homology to plant 12-oxophytodienoate-10,11-reductase gene (*OPR*) (Genbank AFQF01002613) fragments used for the construction of the RNAi constructs were amplified from *F. oxysporum* cDNA by PCR using the primers shown in **Table 1**.

The *FRP1* and *FOW2* fragments were 817 and 846 bp, respectively and were cloned into the pHannibal intermediate RNAi vector in the sense (*XhoI/EcoRI*) and antisense (*BamHI/XbaI*) orientations under the control of the cauliflower mosaic virus 35S and the OCS-terminator. The RNAi cassettes were then excised with *NotI* and cloned into the pUQC247 binary vector. In the case of *OPR*, the fragment was 746 bp long and was cloned into the pKannibal intermediate RNAi vector using *HindIII/XbaI* and *EcoRI/KpnI* for the sense and antisense fragments, respectively and the *NotI* fragment was later transferred to the pART27 binary vector. The RNAi plasmids were transferred to *Agrobacterium tumefaciens* by triparental mating (Vanhaute et al., 1983), and transgenic Arabidopsis plants (Columbia-0 ecotype) generated by *Agrobacterium*—mediated transformation was done using floral dipping (Clough and Bent, 1998). All experiments were carried out using homozygous T3 lines.

QUANTITATIVE REAL-TIME RT-PCR ANALYSIS

Fungal RNA was extracted from the aerial part of Arabidopsis plants 7 days after inoculation as described previously (Purnell and Botella, 2007). Quantitative Real Time- (qRT)-PCR was performed as described by Koia et al. (2012). In summary, first strand cDNA synthesis was conducted using the SuperScript III RT kit (Invitrogen) according to the manufacturer's instructions. qRT-PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems) and the 7900 HT Sequence Detection System (Applied Biosystems). The primer pairs used for qRT-PCR are shown in **Table 2** and were designed using Primer Express software (Applied Biosystems).

Gene expression analysis was performed using SDS Version 2.2.2 software (Applied Biosystems). The results shown are average values from three independently prepared RNA samples.

RESULTS

SELECTION OF FUNGAL GENES FOR DOWN REGULATION AND PRODUCTION OF TRANSGENIC LINES

The purpose of our research was to determine whether the HD-RNAi technology can be used to silence genes in *F. oxysporum*. Although many pathogen genes would be adequate targets for this purpose, the ultimate application of the technology is to produce plants resistant to the disease and it is therefore important to identify genes required for fungal pathogenicity. Forward genetics studies have revealed a number of genes involved in *Fusarium* pathogenicity including argininosuccinate lyase (*ARG1*); class V and class VII chitin synthases (*CHSV* and *CHSVb*); two different heterotrimeric G protein α subunits (*FGA1* and *FGA2*); a heterotrimeric G protein β subunit (*FGB1*), a MAP kinase (*FMK1*) and a mitochondrial carrier protein (*FOW1*) (Di Pietro et al., 2001; Namiki et al., 2001; Inoue et al., 2002; Jain et al., 2002, 2003, 2005; Madrid et al., 2003; Martin-Urdiroz et al., 2008).

For this study we selected three *Fusarium* genes with different characteristics. The first targeted gene, *FOW2* (Genbank AB266616.1) encodes a putative transcription regulator belonging to the Zn(II)2Cys6 family and has been shown to be essential for pathogenicity of *F. oxysporum* f. sp. *melonis* (Imazaki et al., 2007). *FOW2*-targeted mutants completely lost pathogenicity, being unable to colonize the roots of the host but were not impaired in vegetative growth or conidiation when grown in culture. In addition, mutations in the same gene of a different *forma specialis* (f. sp. *lycopersici*) also resulted in loss of pathogenicity, implying the universal importance of the gene for pathogenicity. The second gene selected was *FRP1* (Genbank AY673970.1) which encodes an F-box protein that interacts with SKP1 to facilitate targeting of proteins to the SCF-ubiquitination complex. *FRP1* is necessary for *F. oxysporum* f. sp. *lycopersici* pathogenicity and, as with *FOW2*, *FRP1*-deficient mutants were still able to grow in artificial media but did not colonize host plants (Duyvesteijn et al., 2005). The final target of our study is a gene encoding for a protein with homology to 12-oxo-phytodienoate reductase (*OPR*) (Genbank AFQF01002613), an enzyme involved in the biosynthesis of jasmonic acid (JA) in plants (Wasternack, 2007). It has been recently shown that JA-insensitive *coi1* Arabidopsis mutants display nearly complete resistance to *F. oxysporum* f. sp.

Table 1 | List of primer sequences for cloning RNAi constructs.

<i>FRP1-F</i>	5'-GATCTAGACTCGAGACTTGCCTCCAAATCGTG-3'
<i>FRP1-R</i>	5'-GAGGATCCGAATTCCTATTGAGCCAGAAGTCC-3'
<i>FOW2-F</i>	5'-GCTCTAGACTCGAGAAGTCTGGCTCTAGTGGAAA-3'
<i>FOW2-R</i>	5'-CTGGATCCGAATTCATCTGTTGGGTGCGTATT-3'
<i>OPR-F</i>	5'-AAAGCTTGGTACCAGAAACCGAGGAAGTCCGG-3'
<i>OPR-R</i>	5'-TTCTAGAATTCAGTTCACGACAGAGGTGACTC-3'

Restriction enzyme recognition sequences are underlined.

Table 2 | List of primer sequences for qRT-PCR.

Gene	Forward	Reverse
<i>FRP1</i>	5'-ATCAGCGTCAACCTCCTCCGACT-3'	5'-ATGGTCAAGGGGCC TTAGAGGT-3'
<i>FOW2</i>	5'-TCCAGTCCCAGCTCTGGATCC-3'	5'-AGGTCCAGTGGATGAGCGCCT-3'
<i>OPR</i>	5'-TCGTTGAACGGCGGTATGAGCA-3'	5'-ACATCATTGAATCCGC CAGCGGA-3'
<i>ACTIN</i>	5'-CACCACCTTCAACTCATCA-3'	5'-TCGAGAGAGACCAGGG TACAT-3'

conglutinans and it is believed that *F. oxysporum* might produce JA and use it to target the COI1 protein and to disrupt the host's defense mechanism (Thatcher et al., 2009; Trusov et al., 2009). The JA biosynthetic pathway has not been established in fungi but Genebank searches using the translated Arabidopsis At2g06050 gene (encoding for OPR3) found a homologous gene in chromosome 4 of *F. oxysporum* (Fo5176), which is a variant of *f. sp. conglutinans*, and hereafter we call the gene *OPR*.

A fragment of ~750–850 bp for each of the selected genes was cloned into an intermediate vector in sense and antisense orientations with the PDK intron as a spacer under the control of the Cauliflower mosaic virus 35S promoter (CaMV 35S) and the RNAi cassette was excised and cloned into a binary vector (Wesley et al., 2001) (Supplementary Figure S1). In order to avoid the chances of cross effects of the ectopic siRNAs on plant transcripts in our HD-RNAi studies we selected cDNA fragments with little or no homology with their plant variants. The *F. oxysporum* *FOW2*, *FRP1*, and *OPR* fragments used in our study were used in BLAST searches against the fully sequenced Arabidopsis genome. No homologous sequences for *FOW2* or *FRP1* were found in Arabidopsis neither using high or low stringency parameters. For the *OPR* fragment an overall sequence identity of 43% was found between the fungal and Arabidopsis counterparts. Wild type Arabidopsis ecotype Columbia-0 (Col-0) plants were transformed with the specific RNAi constructs for each of the three targeted genes using *A. tumefaciens* (LBA4404). Ten to fifteen independent transgenic lines were generated for each of the three constructs and 2–3 stable homozygous lines were selected and further characterized. Although RNAi constructs are specifically designed to target gene expression in *F. oxysporum*, it is plausible that off-target effects may occur. We searched the entire Arabidopsis genome for genes with homology to either *FOW2* or *FRP1*, however, we did not find highly similar sequences (data not shown). We also compared the sequences of the Arabidopsis *OPR3* and *F. oxysporum* *OPR* genes. Despite the evident homology at the protein level, the DNA sequences were displayed divergence low level of similarity (43% overall nucleotide identity) (Supplementary Figure S2) preventing a possibility for off-target silencing in transgenic Arabidopsis lines. The resulting Arabidopsis transgenic lines for each *FOW2*-, *FRP1*-, and *OPR*-RNAi did not display any observable phenotypic differences from Col-0 wild type plants in germination, growth parameters and seed production. Importantly, Arabidopsis mutants lacking *OPR3* (*opr3*) are male-sterile; and therefore unable to produce seeds by self-pollination (Stintzi and Browse, 2000). We produced over 30 Arabidopsis *OPR*-RNAi transgenic lines, all of which produced WT looking siliques and subsequently seeds, therefore confirming that there were no off-target gene silencing effects in the Arabidopsis HD-RNAi transgenic lines.

HD-RNAi RESULTS IN DOWN-REGULATION OF TARGETED GENES IN *F. OXYSPORUM* INFECTING TRANSGENIC ARABIDOPSIS LINES

To determine whether HD-RNAi results in down-regulation of the targeted *F. oxysporum* genes we measured the relative expression levels for all three genes in fungi infecting their corresponding transgenic lines and compared them with the levels present

in fungi infecting wild type Arabidopsis plants. For this purpose, total RNA was extracted from above-ground tissues of infected plants 7 days after inoculation with the pathogen. At this stage, fungal mycelium is abundant in the vasculature of the host plant leaves and stems. Fungal gene expression levels were determined by quantitative real time PCR (qRT-PCR) with gene-specific primers designed to bind outside the DNA fragments used for the RNAi constructs to avoid possible artifacts. Levels of *F. oxysporum* actin (GenBank JQ965663.1) were assumed to remain constant in all fungal cells and were therefore used for normalization of results. Optimization experiments showed that all primer pairs were able to amplify their intended specific targets, confirmed by sequencing, with reproducible Ct values (results not shown).

Figure 1 shows that for each of the three targeted genes, the mRNA levels in fungi infecting their respective Arabidopsis HD-RNAi lines are lower than those observed in fungi infecting Col-0 controls. *FOW2* mRNA levels were reduced by 75 and 78% in the two independent transgenic lines assayed (**Figure 1A**). Meanwhile, *FRP1* levels were reduced by 90 and 76% in the two transgenic lines assayed (**Figure 1B**). Down-regulation of *OPR* expression levels was variable with a reduction in mRNA levels of 59, 75, and 83% in the three transgenic lines studied (**Figure 1C**). It is important to remark that, even though in some cases the reduction in mRNA levels was quite dramatic, no complete silencing was achieved for any of the targeted genes.

DISEASE PROGRESSION IN HD-RNAi TRANSGENIC ARABIDOPSIS LINES

Even though *FOW2* mRNA levels in fungi infecting transgenic *FOW2* HD-RNAi lines was reduced to 22–28% of the normal expression levels detected in fungi infecting wild type plants, the effect on disease progression measured by the number of yellow (chlorotic) leaves per plant was not as dramatic (**Figure 2A**). Only one transgenic line, *FOW2*-RNAi-6, showed a consistent and statistically significant decrease in yellow leaves counts at the advanced infection stages, indicating a slow-down in the disease progression (**Figure 2A**). Nevertheless, although the disease progression was not radically slowed, the percentage of surviving plants at 18 days-post inoculation was significantly improved in both transgenic lines (*FOW2*-RNAi-6 and *FOW2*-RNAi-11) to more than double of number observed in wild type plants (**Figure 2B**).

A very different behavior was observed in the transgenic lines targeting the fungal *FRP1* gene. Disease progression was clearly slowed down in the transgenic lines compared to wild type controls, with an statistically significant reduction in the number of yellow leaves observed for both transgenic lines at the advanced stages of infection (**Figure 3A**). As a consequence, survival rates were greatly enhanced in the transgenic lines with 30 and 50% surviving fungal infection in lines *FRP1*-RNAi-24 and *FRP1*-RNAi-40 respectively, compared to 10% in wild type plants (**Figure 3B**).

Targeting of the *F. oxysporum* *OPR* gene produced the best results in terms of disease symptom progression with all three studied transgenic lines (*OPR*-RNAi-7, *OPR*-RNAi-8, and *OPR*-RNAi-9) producing significantly lower numbers of yellow leaves than wild type plants from the very early stages of infection

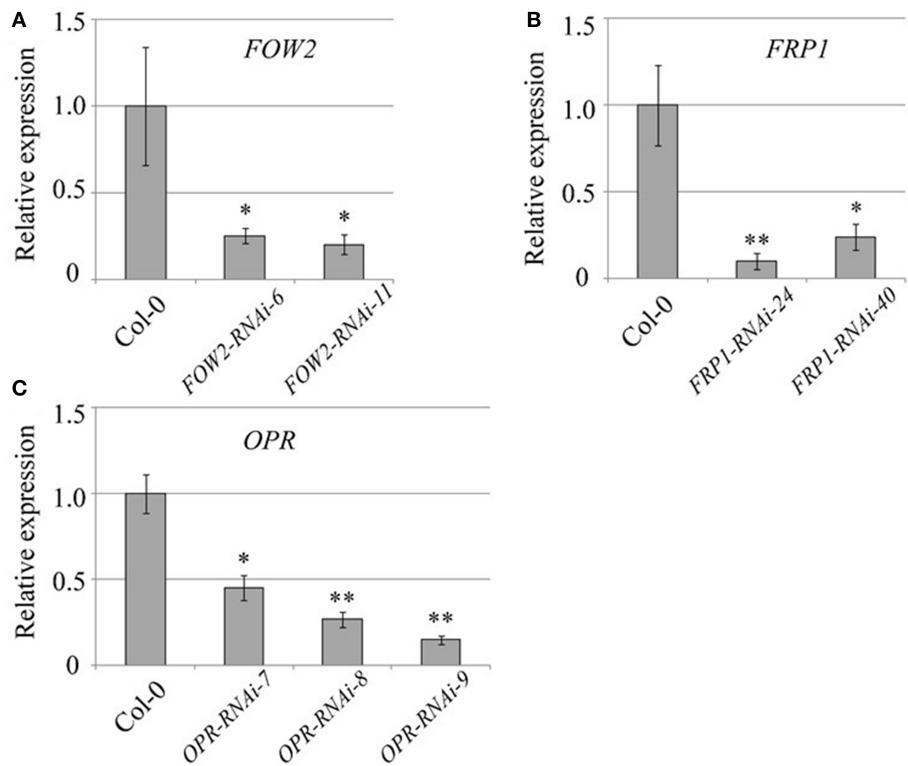


FIGURE 1 | Quantification of *Fusarium oxysporum* mRNA levels. Wild type and T3 homozygous transgenic lines expressing the HD-RNAi constructs for **(A)** *FOW2*, **(B)** *FRP1*, and **(C)** *OPR* were infected with *F. oxysporum* f. sp. *conglutinans*. Total RNA extracted from above-ground tissues of 3-week-old transgenic lines at 7 days post inoculation was used ($n = 20$) for cDNA synthesis as a template for quantitative real

time PCR with pathogen gene-specific primers. *F. oxysporum* actin levels (GenBank JQ965663.1) were used for normalization purposes. For each gene, the relative mRNA level measured in *F. oxysporum* infecting wild type plants was given the arbitrary value of one and the remaining mRNA levels referred to it. Values shown are means $\pm$ SE of three biological replicates. * $p \leq 0.05$; ** $p \leq 0.005$.

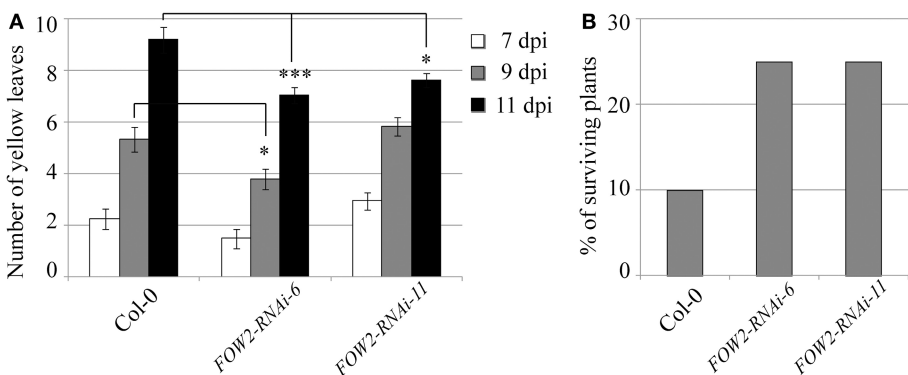
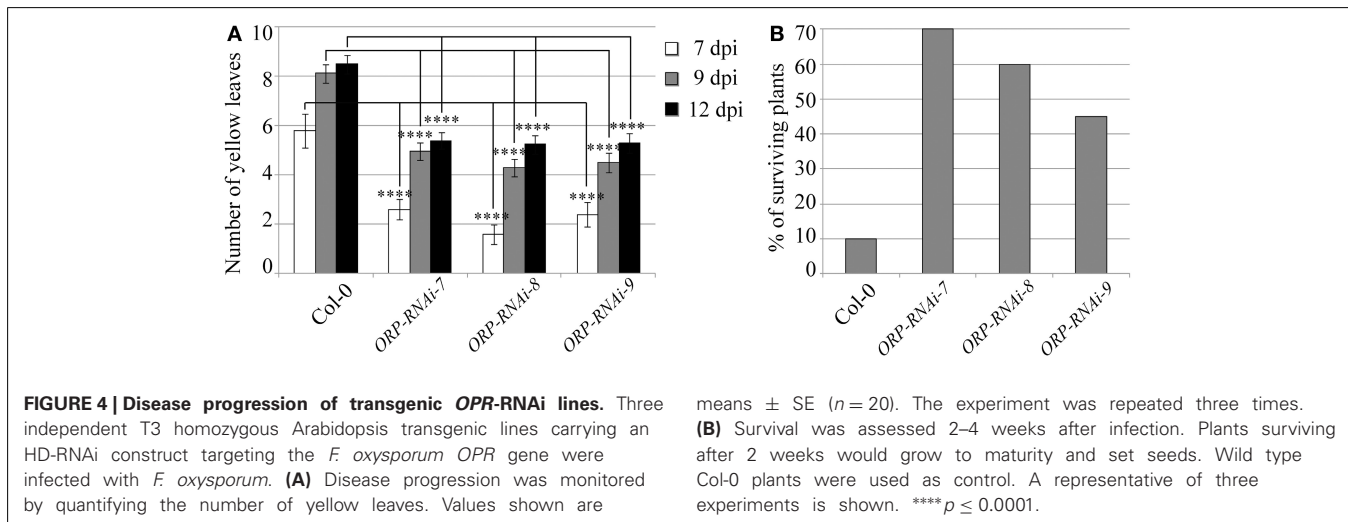
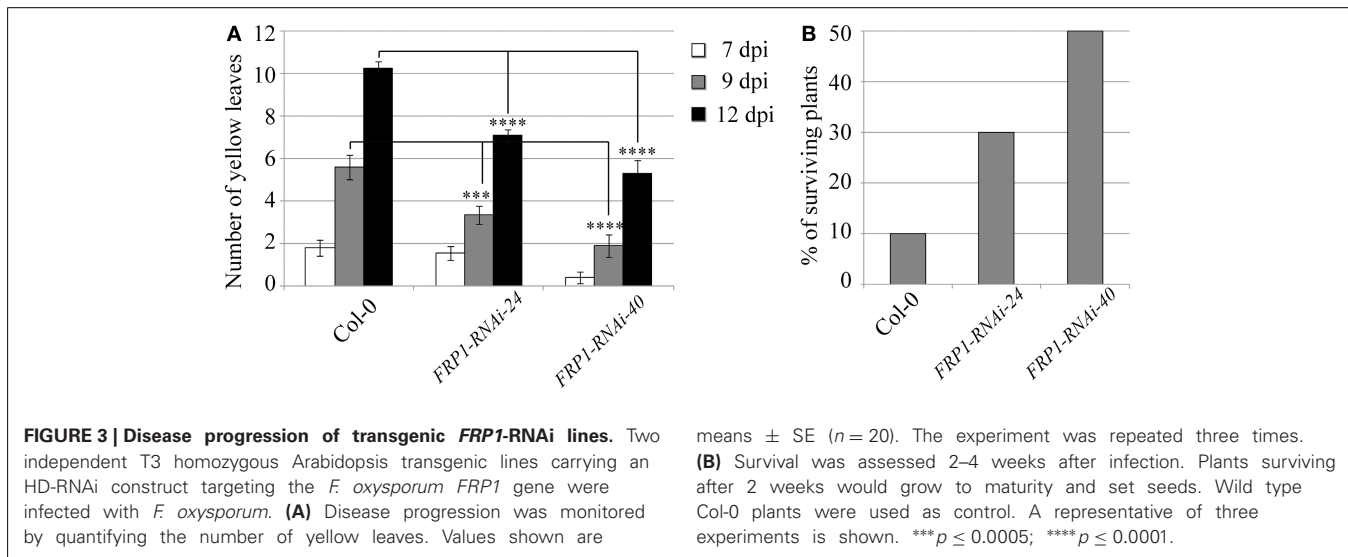


FIGURE 2 | Disease progression of transgenic *FOW2*-RNAi lines. Two independent T3 homozygous *Arabidopsis* transgenic lines carrying an HD-RNAi construct targeting the *F. oxysporum* *FOW2* gene were infected with *F. oxysporum*. **(A)** Disease progression was monitored by quantifying the number of yellow leaves. Values shown

are means $\pm$ SE ($n = 20$). The experiment was repeated three times. **(B)** Survival was assessed 2–4 weeks after infection. Plants surviving after 2 weeks would grow to maturity and set seeds. Wild type Col-0 plants were used as control. A representative of three experiments is shown. * $p \leq 0.05$; *** $p \leq 0.0005$.

(Figure 4A). In addition, the survival rates were quite high with 70, 60, and 45% for transgenic lines *OPR*-RNAi-7 and *OPR*-RNAi-8 and *OPR*-RNAi-9 respectively compared to the 10% survival rate for wild type controls (Figure 4B). A complete

time-lapse sequence showing the disease development in the three transgenic *OPR* lines is shown in Figure 5. Plants surviving infection after 20 days would produce flowers and set seeds.



DISCUSSION

In this work, we have shown that expression of dsRNA molecules targeting pathogen genes in transgenic *Arabidopsis* plants can effectively reduce the mRNA levels of three different genes in *F. oxysporum* infecting the transgenic lines. The level of down regulation achieved in the different lines was substantial, with an average of 75, 83, and 72% for *FWO2*, *FRP1*, and *OPR*, respectively. Two selected transgenic *FWO2*-RNAi lines displayed elevated levels of resistance against *F. oxysporum* infection with slowed disease progression and improved survival rates compared to wild type controls (**Figures 2A,B**). Two *FRP1*-RNAi transgenic lines as well showed clearly delayed disease symptom development as compared to wild type and as expected it resulted in the enhanced survival rate (**Figures 3A,B**). The expression level of *F. oxysporum* *OPR* gene was more variable between transgenic *OPR*-RNAi lines it fed on, perhaps due to the fact that the normal expression levels were quite low compared to the other two genes, hence getting closer to the qRT-PCR detection limit. However, all three transgenic *OPR*-RNAi lines tested were evidently more

resistant to *F. oxysporum* than wild type plants, indicated from the reduced yellow leaf symptoms in all the transgenic lines and markedly increased survival (**Figures 4A,B**).

FWO2 has been proven to be important for pathogenicity in two different *F. oxysporum* forma specialis, *f. sp. melonis* and *f. sp. lycopersici* and it has been suggested that it encodes a transcription regulator controlling the infection competency of *F. oxysporum* pathogens (Imazaki et al., 2007). *FWO2*-defective mutants completely lost their ability to invade plant roots and colonize the plant, even when the pathogen was manually injected into the plant tissues. The fact that we observe relatively mild, although statistically significant, differences in disease progression between the wild type control and the transgenic *FWO2*-RNAi lines might be due to the incomplete silencing achieved in the fungi. mRNA levels were reduced by 75 and 78% in transgenic lines but it is possible that the residual ~25% is enough for *FWO2* to confer pathogenicity, and thus stronger silencing is needed in order to observe a more prominent impact on disease progression. An alternative explanation is that *FWO2* might function largely at

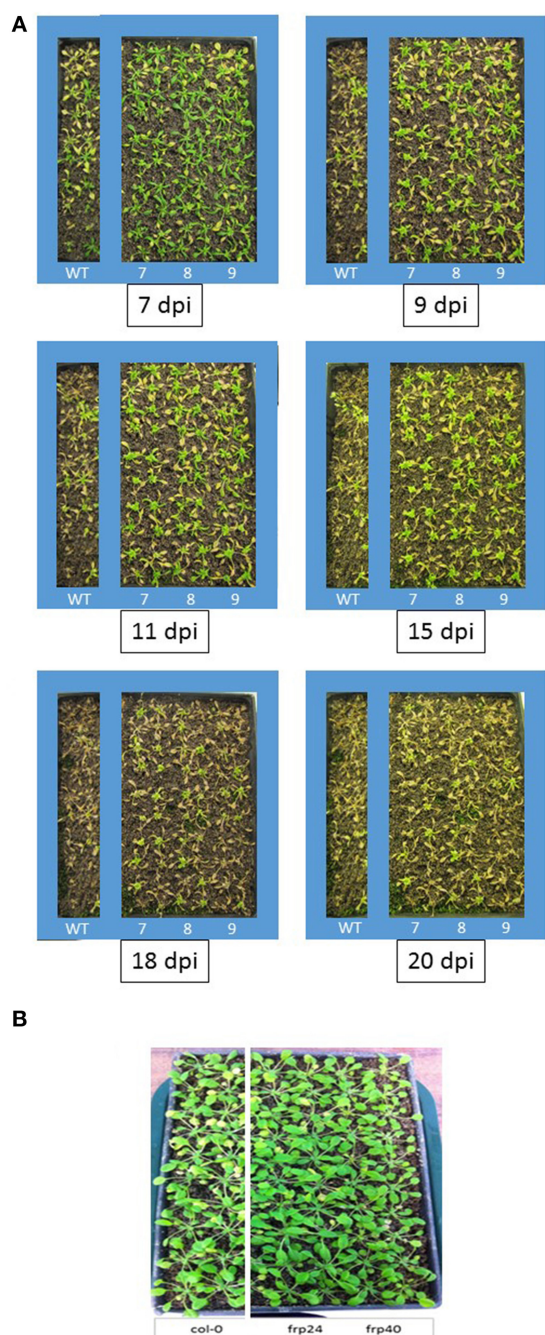


FIGURE 5 | Time-lapse images and disease progression in transgenic *OPR*-RNAi and *FRP1*-RNAi lines. (A) Three independent T3 homozygous Arabidopsis *OPR* transgenic lines (#7, #8, and #9) carrying an HD-RNAi construct targeting the *F. oxysporum* *OPR* gene were infected with *F. oxysporum*. Disease progression was photographed 7, 9, 11, 15, 18, and 20 days after inoculation. Plants surviving after 2 weeks would grow to maturity and set seeds. Wild type Col-0 plants were used as control. A representative of three experiments is shown. **(B)** Two independent T3 homozygous Arabidopsis *FRP1* transgenic lines (#24 and #40) carrying an HD-RNAi construct targeting the *F. oxysporum* *FRP1* gene were infected with *F. oxysporum*. Disease progression was photographed 9 days after inoculation.

the onset of the infection process, perhaps involved in detection of the potential host, while RNAi silencing can only occur after the fungus is established in the host plant. Therefore, the silencing might come a bit late to exert a full impact on the pathogen. Nevertheless, the substantial increase in survival rates observed in both transgenic lines, suggests that the levels of down-regulation achieved for *FWW2* do have an effect on the ability of the pathogen to colonize the plant.

FRP1 is essential for pathogenicity in *F. oxysporum* f. sp. *lycopersici* and it is highly conserved in f. sp. *conglutinans*. Disruption in *FRP1* led to a complete loss of pathogenicity in tomato with mutant fungi unable to colonize the roots (Duyvesteijn et al., 2005). The fact that *FRP1* is an F-box protein and that it physically interacts with *F. oxysporum* SKP1 (Genebank AAT85970.1) suggests that *FRP1* is part of a SCF ubiquitin kinase complex. The *F. oxysporum* f. sp. *lycopersici* *FRP1* protein has highly homologous counterparts (86–100% identity) in other *Fusarium* species (*F. oxysporum* f. sp. *conglutinans*; f. sp. *cubense* races 1 and 4, *F. fujikuroi*, *F. graminearum* and *F. pseudograminearum*) and it is also present in other plant pathogenic fungi such as *Colletotrichum graminicola* (63% identity) and even non plant pathogenic fungi such as *Beauveria bassiana* (66% identity) and *Neurospora crassa* (55% identity). The relatively high level of homology is probably due to the conservation of the F-box motif and points to the suggestion that an *FRP1*-associated SCF ubiquitin ligase complex plays an important role in degradation of pathogenicity-related proteins through ubiquitination (Duyvesteijn et al., 2005). Even though it has been suggested that *FRP1* plays a crucial role in the early events of the *Fusarium* infection process, in our hands, partial silencing of the *FRP1* gene in fungi infecting transgenic Arabidopsis *FRP1*-RNAi lines led to a statistically significant delay in disease symptoms and a marked increase in survival rates. Therefore, in addition to its importance during the first steps of infection, *FRP1* must have additional role/s in the colonization of the plant tissue.

JA is a well-known phytohormone involved in defense and is particularly important against necrotrophic pathogens (Nickstadt et al., 2004; Glazebrook, 2005). Studies in Arabidopsis have shown that overexpression of defense-related proteins in the JA pathway, such as TH12.1, ERF1, and ERF2, confer enhanced resistance to *F. oxysporum* (Epple et al., 1997; McGrath et al., 2005; Berrocal-Lobo and Molina, 2008). In addition, T-DNA knock-outs of AtMYC2, a negative regulator of JA-ethylene defense responsive genes also results in enhanced *F. oxysporum* resistance (Anderson et al., 2004; Trusov et al., 2009; Trusov and Botella, 2012), suggesting that the JA-mediated pathway plays a defense role against *F. oxysporum*. Strikingly, it was recently discovered that mutants lacking the JA receptor COI1, display increased resistance to *F. oxysporum* while mutants defective in JA biosynthesis displayed wild type levels of resistance (Thatcher et al., 2009; Trusov et al., 2009). These results indicate that even though JA generally mediates immunity, it is highly possible that the COI1-mediated signaling pathway contributes to susceptibility to *F. oxysporum* (Thatcher et al., 2009; Trusov et al., 2009). Notably, it has been established that fungi also produce JA and various other jasmonates with over 20 jasmonate compounds identified

in *F. oxysporum* f. sp. *matthioli* (Miersch et al., 1999; Wasternack, 2007). It has been therefore hypothesized that *F. oxysporum* might use its own JA to target COI1 and hijack the host's defense mechanism (Thatcher et al., 2009).

In plants JA is synthesized from α -linolenic acid in a number of reactions catalyzed by lipoxygenases (LOXs), allene oxide synthases (AOSs), and allene oxide cyclases (AOCs) to form 12-oxo-phytodienoic acid (OPDA), which is then converted to JA via 12-oxo-phytodienoate reductases (OPRs) and β -oxidation (Wasternack, 2007). Although the biosynthetic pathways and functions of fungal jasmonates remain elusive, it is suggested that the JA biosynthetic pathway between plants and fungi may be similar (Brodhun et al., 2013). In fact, the pathogenic fungus *Lasiodiplodia theobromae* produced OPDA and an exogenous supplement of synthetic linolenic acid led to jasmonate production (Tsukada et al., 2010), although the presence of JA biosynthetic enzymes (like LOX and AOS) in this fungus was not confirmed (Brodhun et al., 2013). Remarkably, a study characterizing the enzymatic properties of a putative LOX in *F. oxysporum* f. sp. *lycopercisi*, showed that it was similar to plant LOXs, involving iron instead of manganese at the active site (Brodhun et al., 2013).

Following on the hypothesis that JA produced by the fungus could play an important role in overcoming the plant defense, we hypothesized that total or partial silencing of the *OPR* gene could have an effect on the ability of *F. oxysporum* to infect the plant. Our results unambiguously demonstrate that the level of silencing achieved by HD-RNAi in our experiments was sufficient to significantly delay the progression of the disease. Although all genotypes experienced severe stress upon infection with the pathogen, the transgenic lines displayed greatly reduced disease symptoms and a much higher survival rate compared to wild type controls. We suggest that the *OPR*-RNAi transgenic plants were better equipped to withstand the pathogen probably due to down-regulation of the *F. oxysporum* *OPR* gene and subsequent reduction in jasmonate synthesis by the attacking fungus.

In summary, our results show that HD-RNAi can effectively down-regulate endogenous genes in pathogenic hemi-biotrophs although the degree of silencing is variable depending on the gene targeted and the transgenic line examined. No silencing was observed in a sequence-unrelated fungal gene (Supplementary Figure S3). No complete silencing has been achieved but partial silencing was enough to at least delay the disease progression and increase the rate of survival. HD-RNAi in the Arabidopsis-*F. oxysporum* interaction system could be used to quickly test fungal genes suspected to be involved in pathogenicity. In agricultural terms, achieving a delay in disease progression can have a very practical application as it provides the farmers with extra time to diagnose and treat the pathogen, minimizing crop losses. Furthermore, production of HD-RNAi constructs targeting multiple virulence-related genes should provide more durable resistance than methods relying on a single gene.

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Chicoric acid: chemistry, distribution, and production

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Though chicoric acid was first identified in 1958, it was largely ignored until recent popular media coverage cited potential health beneficial properties from consuming food and dietary supplements containing this compound. To date, plants from at least 63 genera and species have been found to contain chicoric acid, and while the compound is used as a processing quality indicator, it may also have useful health benefits. This review of chicoric acid summarizes research findings and highlights gaps in research knowledge for investigators, industry stakeholders, and consumers alike. Additionally, chicoric acid identification, and quantification methods, biosynthesis, processing improvements to increase chicoric acid retention, and potential areas for future research are discussed.

Keywords: phenolics, polyphenolics, cichoric acid, caffeic acid derivative, dicaffeoyltartaric acid, hydroxycinnamic acid, phenolic acid

INTRODUCTION

Recent US consumer interest in boosting their dietary intake of chicoric acid followed popular-media coverage of claims that consumption of products containing chicoric acid had promising health benefits (Drazen, 2003). A recent literature search on chicoric acid reveals, not unsurprisingly, that a preponderance of the published research on chicoric acid is related to its potential medicinal uses (>50%) and research related to its chemistry (~18%), natural production in agriculture (~13%), and retention in foods (~18%) is lagging. Improved knowledge relating to chicoric acid biochemistry, how to enhance it in plant production, and how to retain its presence and activity in food and food products is needed.

In 1958, while working on the leaves of chicory (*Cichorium intybus* L.) plants, Scarpati and Oriente (1958) isolated and identified a phenolic compound that was a tartaric acid ester of two caffeic acids (a hydroxycinnamic acid; **Figure 1**); they proposed naming it chicoric acid. Since that first discovery, chicoric acid has since been charted in many plant families, including those of seagrass, horsetail, fern, lettuce, and basil (**Table 1**). Other common names for chicoric acid are cichoric acid and dicaffeoyltartaric acid (Lee and Scagel, 2009), but for conciseness we will refer to this compound as chicoric acid.

Reports have indicated that chicoric acid helps a plant protect itself from insects and infection from viruses, bacteria, fungi, and nematodes (Cariello and Zanetti, 1979; Rees and Harborne, 1985; Snook et al., 1994; Nishimura and Satoh, 2006); and that it aids in wound healing in plants after mechanical damage (Tomas-Barberan et al., 1997). Beyond the better understood roles it has in seed germination, additional research is needed to better understand why plants produce chicoric acid and to corroborate theories that phenolic acids defend against microbial and herbivore attack by acting as deterrents, toxins,

or signaling molecules (Harborne, 1979; Gallagher et al., 2010; Mandal et al., 2010). General summaries of phenolics' roles in ecosystem (Hattenschwiler and Vitousek, 2000) and ecophysiology (Cheynier et al., 2013) are available.

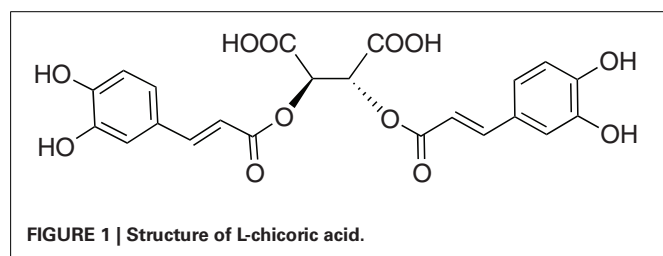
The significance of plant phenolics to humans is still developing. While individual phenolics have been used in plant systematics and have become recognized for their utility in quality monitoring of crops and product manufacturing, they are now being investigated for possible human health benefits. A thorough identification of phenolics in foods will aid research in determining food and dietary supplement authenticity, quality assurance standards, and health investigations (Winter and Herrmann, 1986; Stuart and Wills, 2003; Lee, 2010, 2014; Lee and Scagel, 2009, 2010; Sanzini et al., 2011). With our current awareness in eating a healthy diverse diet, and the recent attention chicoric acid has received, we consider this review necessary to summarize the present data on plant sources of chicoric acid and factors that may influence their identification, production, and use.

Our objective was to compile scientific findings on chicoric acid to aid future work: including its recognition, its known prevalence within the plant kingdom, its distribution throughout a plant, growing condition effects upon it, and processing techniques to retain its quality within final products.

IDENTIFICATION, SYNTHESIS, AND BIOSYNTHESIS CHICORIC ACID

OBTAINABILITY, SYNTHESIS, AND FORMS

Chicoric acid can be obtained from isolated and purified plant materials (**Table 1**) or synthesized (Scarpati and Oriente, 1958; Synoradzki et al., 2005). Pure chicoric acid is a white powder (Synoradzki et al., 2005; personal observation), and now available as a purified standard via numerous chemical companies (e.g.,



Cerilliant Chemical Company, Indofine Chemical Company, Inc., Sigma-Aldrich Co, LLC, etc.). The most abundant natural form is L-chicoric acid [i.e., (-)-chicoric acid, 2,3-dicaffeoyl-L-tartaric acid, 2,3-O-dicaffeoyltartaric acid, 2R,3R-O-dicaffeoyltartaric acid, or di-*E*-caffeoyl-(2R-3R)-(-)-tartaric acid] and has been reported in the majority of the plants listed in **Table 1**, but the stereoisomer *meso*-chicoric acid (i.e., dicaffeoyl-*meso*-tartaric acid or di-*E*-caffeoyl-(2R-3S)-(-)-tartaric acid) has also been reported at a lesser levels in horsetail sprouts (*Equisetum arvense* L.) (Veit et al., 1991, 1992; Hohlfield et al., 1996), iceberg lettuce (*Lactuca sativa* L.) (Baur et al., 2004; Luna et al., 2012), and purple coneflower (*Echinacea purpurea* L. Monech) (Perry et al., 2001).

Identification of L-chicoric acid and *meso*-chicoric acid in plant samples has not been consistent. L-chicoric acid and an isomer (unknown form) were reported in chicory leaves (Heimler et al., 2009). Both L-chicoric acid and *meso*-chicoric acid were found in iceberg lettuce (Baur et al., 2004; Luna et al., 2012). *Equisetum arvense* L. fertile sprouts and gametophytes contained *meso*-chicoric acid and no L-chicoric acid (Veit et al., 1992), but Hasegawa and Taneyama (1973) reported only L-chicoric acid in *E. arvense* L. (unspecified growth stage of tissue). Hasegawa and Taneyama (1973) found L-chicoric acid in numerous fern frond samples (**Table 1**), though Veit et al. (1992) reported no L-chicoric acid and only *meso*-chicoric acid in their fern samples.

In some cases the *meso*-chicoric acid identified from plant extracts may have been due to isomerization of L-chicoric acid after sample extraction and/or purification (Snook et al., 1994; Perry et al., 2001). Snook et al. (1994) showed that a small amount of L-chicoric acid in peanut leaf terminal methanol extract isomerized into *meso*-chicoric acid at room temperature. Although three accounts from a single research group confirmed by Nuclear Magnetic Resonance (NMR) the tartaric acid structure of purified *meso*-chicoric acid of horsetail barren sprouts (Veit et al., 1991, 1992; Hohlfield et al., 1996), an independent report found L-chicoric acid, but no *meso*-chicoric acid in horsetail (Hasegawa and Taneyama, 1973).

Differences in chicoric acid forms among research reports may be a result of the methods used for sample extraction and identification. L-chicoric acid and its isomers can be distinguished by 2D-paper chromatography. However, separation via High Performance Liquid Chromatography (HPLC), L- and D-chicoric acids (if D-chicoric acid exists) co-elute (Williams et al., 1996; Baur et al., 2004). In some cases, *meso*-chicoric acid was found to elute immediately after L-chicoric acid (Snook et al., 1994; Baur et al., 2004) in reversed-phase HPLC conditions. Thin Layer Chromatography (TLC) separates L-chicoric acid from *meso*-chicoric acid (Veit et al., 1991, 1992). Phenolic acid conjugated

to *meso*-tartaric acid is rarely reported, but *p*-coumaroyl-*meso*-tartaric acid has been found in spinach (Winter and Herrmann, 1986; Bergman et al., 2001), indicating *meso*-tartaric acid conjugation to phenolic acids can occur naturally, however, it has yet to be confirmed that it formed before extraction. Work is still needed to confirm if *meso*-chicoric acid is present naturally.

IDENTIFICATION AND QUANTIFICATION

Chicoric acid identification and quantification can be achieved by either Planar (Paper or Thin Layer) Chromatography (Scarpati and Oriente, 1958; Rees and Harborne, 1985; Nicolle et al., 2004), HPLC coupled with a Diode Array Detector (DAD) and/or Mass Spectrometer (MS) Detector, or NMR spectroscopy (Lee and Scagel, 2009; Nuissier et al., 2010; Juskiewicz et al., 2011; Ritota et al., 2013).

Chicoric acid spectra by NMR can be found in Nuissier et al. (2010). Infrared spectra of chicoric acid can be found in Scarpati and Oriente (1958). If a purified chicoric acid standard is unavailable, than an extract of *E. purpurea*, chicory, or dandelion (since it is their main phenolic) can be made to aid peak identification (Williams et al., 1996; Lee and Scagel, 2009, 2010; Juskiewicz et al., 2011; and additional references listed in **Table 1**).

A clear UV-visible spectra (maximum absorption at 330 with a 300 nm shoulder) (Scarpati and Oriente, 1958; Pellati et al., 2005; Lee and Scagel, 2009) and a HPLC chromatogram of chicoric acid, and other caffeic acid derivatives, elution is available in past works (Bergeron et al., 2000; Kim et al., 2000; Baur et al., 2004; Nicolle et al., 2004; Lee and Scagel, 2009). A HPLC method (INA-Institute for Nutraceutical Advancement method 106.000) from NSF (National Science Foundation) International (Ann Arbor, MI, USA) is accessible as well. Clear chicoric acid mass spectra examples can be found in Shiga et al. (2009) and Mulinacci et al. (2001); mother and fragmented masses have been reported in numerous papers with various MS settings, so one of these references can be used for comparison with a comparable analysis (Mulinacci et al., 2001; Baur et al., 2004; Lee and Scagel, 2009; Shiga et al., 2009; Pellati et al., 2011; Ribas-Agusti et al., 2011; Carazzone et al., 2013).

Capillary electrophoresis (with DAD) has also been evaluated in chicoric acid quantification in *E. purpurea* dried press juice (Manèk and Kreft, 2005). The ability to predict chicoric acid production in *E. purpurea* plants by an extrapolation model using DNA fingerprinting and HPLC concentration results has also been explored (Baum et al., 2001). Developing rapid and accurate techniques for chicoric acid identification and quantification as well new methods for authenticating and predicting production in plants will aid future work by researchers, agronomists, and manufacturers.

EXTRACTION

Sample preparation has often been overlooked in quality analysis research, even though as the first laboratory stage of chemical analysis it has great consequences for the results (Williams et al., 1996; Bergeron et al., 2000; Stuart and Wills, 2000; Perry et al., 2001; Lee and Scagel, 2009; Kim and Verpoorte, 2010; Lee et al., 2012). Sample preparation can impact not only accurate identification of chicoric acid (e.g., L- vs. *meso*- form of chicoric

Table 1 | Chicoric acid has been identified in the following plants.

Common name(s)	Order	Family	Genus and species	Plant parts evaluated and chicoric acid found in	References
Burhead, Chapau-de-couro	Alismatales	Alismataceae	<i>Echinodorus grandiflorus</i>	Leaves	Garcia Ede et al., 2010
Threelobe beggarticks, trifid burr marigold, marigold burr	Asterales	Asteraceae	<i>Bidens tripartita</i> L.	Aerial part	Pozharitskaya et al., 2010
Endive, cultivated endive, escarole	Asterales	Asteraceae	<i>Cichorium endivia</i> L.	Aerial part	Winter and Herrmann, 1986; Goupy et al., 1990; Degl'Innocenti et al., 2008; Mascherpa et al., 2012
Chicory, radicchio	Asterales	Asteraceae	<i>Cichorium intybus</i> L.	Green/red and etiolated aerial part, roots, and seeds	Scarpati and Oriente, 1958, Bridle et al., 1984; Rees and Harborne, 1985; Winter and Herrmann, 1986; Chkhikvishvili and Kharebava, 2001; Mulinacci et al., 2001; Innocenti et al., 2005; Qu et al., 2005; Rossetto et al., 2005; Heimler et al., 2009; Jaiswal et al., 2011; Juskiewicz et al., 2011; Carazzone et al., 2013; Ritota et al., 2013; Ziamajidi et al., 2013
Smooth hawkbeard	Asterales	Asteraceae	<i>Crepis capillaris</i> L. Wallr.	Flower heads	Zidorn et al., 2005
Smooth purple coneflower	Asterales	Asteraceae	<i>Echinacea atrorubens</i> Nutt.	Aerial parts (flower heads, leaves, stems) and roots	Binns et al., 2002
Echinacea, black Samson Echinacea	Asterales	Asteraceae	<i>Echinacea augustifolia</i> DC.	Aerial part, roots, and pills (unknown parts)	Chkhikvishvili and Kharebava, 2001; Perry et al., 2001; Binns et al., 2002; Laasonen et al., 2002; Brown et al., 2010
Topeka purple coneflower	Asterales	Asteraceae	<i>Echinacea laevigata</i> (C.L. Boynt. and Beadle) S.F. Blake	Aerial parts (flower heads, leaves, stems) and roots	Binns et al., 2002; Pellati et al., 2005
Echinacea, pale purple coneflower, coneflower	Asterales	Asteraceae	<i>Echinacea pallida</i> Nutt.	Aerial parts and roots	Perry et al., 2001; Binns et al., 2002; Laasonen et al., 2002; Pellati et al., 2005; Brown et al., 2010; Sabra et al., 2012; Thomsen et al., 2012
Bush's purple coneflower	Asterales	Asteraceae	<i>Echinacea paradoxa</i> (J.B.S. Norton) Britton	Roots	Binns et al., 2002; Pellati et al., 2005
Echinacea, eastern purple coneflower, coneflower, purple coneflower	Asterales	Asteraceae	<i>Echinacea purpurea</i> L. Monech	Aerial parts (flower heads, leaves, stems) and roots	Wills and Stuart, 1999, 2000; Bergeron et al., 2000, 2002; Kim et al., 2000; Perry et al., 2001; Laasonen et al., 2002; Stuart and Wills, 2003; Pellati et al., 2005, 2011; Liu et al., 2006; Wu et al., 2007a; Araim et al., 2009; Lee and Scagel, 2009, 2010; Brown et al., 2010; Chen et al., 2010; Lee, 2010; Zhang et al., 2011; Sabra et al., 2012; Thomsen et al., 2012

(Continued)

Table 1 | Continued

Common name(s)	Order	Family	Genus and species	Plant parts evaluated and chicoric acid found in	References
Sanguin purple coneflower, purple coneflower	Asterales	Asteraceae	<i>Echinacea sanguinea</i> Nutt.	Aerial parts (flower heads, leaves, stems) and roots	Binns et al., 2002; Pellati et al., 2005
Wavyleaf purple coneflower	Asterales	Asteraceae	<i>Echinacea simulata</i> R.L. McGregor	Aerial parts (flower heads, leaves, stems) and roots	Binns et al., 2002; Pellati et al., 2005
Tennesseeensis purple coneflower	Asterales	Asteraceae	<i>Echinacea tennesseeensis</i> (Beadle) Small	Aerial parts (flower heads, leaves, stems) and roots	Binns et al., 2002; Pellati et al., 2005
Hairy cat's ear	Asterales	Asteraceae	<i>Hypochaeris radicata</i> L.	Flowering heads	Zidorn et al., 2005
Indian lettuce	Asterales	Asteraceae	<i>Lactuca indica</i> L.	Aerial parts	Kim et al., 2010
Iceberg lettuce, loose leaf lettuce, romaine lettuce, butterhead lettuce, baby lettuce	Asterales	Asteraceae	<i>Lactuca sativa</i> L.	Lettuce head (edible and inedible parts) and leaves	Winter and Herrmann, 1986; Tomas-Barberan et al., 1997; Gil et al., 1998; Cantos et al., 2001; Romani et al., 2002; Baur et al., 2004; Becker et al., 2004, 2013; Nicolle et al., 2004; Beltran et al., 2005; Kenny and O'Beirne, 2009; Oh et al., 2009; Chisari et al., 2010; Mulabagal et al., 2010; Jaiswal et al., 2011; Ribas-Agusti et al., 2011; Luna et al., 2012; Abu-Reidah et al., 2013; Mai and Glomb, 2013
Fall dandelion	Asterales	Asteraceae	<i>Leontodon autumnalis</i> L.	Flower head	Grass et al., 2006
Spiny sowthistle	Asterales	Asteraceae	<i>Sonchus asper</i> L. Hill	Leaves	Gatto et al., 2011
Common sowthistle, smooth sowthistle	Asterales	Asteraceae	<i>Sonchus oleraceus</i> L.	Leaves	Gatto et al., 2011; Ou et al., 2012
Common dandelion, dandelion	Asterales	Asteraceae	<i>Taraxacum officinale</i> F.H. Wigg.	Flowers, involucre bracts, leaves, stems, and roots	Williams et al., 1996; Chkhikvishvili and Kharebava, 2001; Schütz et al., 2005; Gatto et al., 2011; Park et al., 2011
Zucchini	Cucurbitales	Cucurbitaceae	<i>Cucurbita pepo</i> L.	Fruit	Iswaldi et al., 2013
Horsetail	Equisetales	Equisetaceae	<i>Equisetum</i> hybrids: <i>Equisetum arvense</i> × <i>palustre</i> <i>Equisetum</i> × <i>litorale</i> <i>Equisetum</i> × <i>rothmaleri</i> <i>Equisetum</i> × <i>torgesianum</i> act. non Rothm.	Part not specified	Veit et al., 1992
Field horsetail, common horsetail	Equisetales	Equisetaceae	<i>Equisetum arvense</i> L.	Sprouts (fertile) and gametophytes	Hasegawa and Taneyama, 1973; Veit et al., 1991, 1992; Hohlfeld et al., 1996
Scouringrush horsetail	Equisetales	Equisetaceae	<i>Equisetum hyemale</i> L.	Frond	Hasegawa and Taneyama, 1973

(Continued)

Table 1 | Continued

Common name(s)	Order	Family	Genus and species	Plant parts evaluated and chicoric acid found in	References
Causasian whortleberry	Ericales	Ericaceae	<i>Vaccinium arctostaphylos</i> L.	Leaves, fruits	Chkhikvishvili and Kharebava, 2001
Peanut	Fabales	Fabaceae	<i>Arachis hypogaea</i> L.	Leaf terminals	Snook et al., 1994
Snakeroot, serpentine roots, chandra	Gentianales	Apocyanaceae	<i>Rauvolfia serpentine</i> (L.) Benth. Ex Kurz.	Shoots and roots.	Nair et al., 2013
Sickle seagrass, thalassia	Hydrocharitales	Hydrocharitaceae	<i>Thalassia hemprichii</i> Asch.	Leaves	Qi et al., 2012
Borage, starflower, common borage	Lamiales	Boraginaceae	<i>Borago officinalis</i> L.	Leaves	Gatto et al., 2011
Basil, sweet basil	Lamiales	Lamiaceae	<i>Ocimum basilicum</i> L.	Aerial parts and roots	Lee and Scagel, 2009, 2010; Shiga et al., 2009; Lee, 2010; Nguyen et al., 2010; Kwee and Niemeyer, 2011; Scagel and Lee, 2012
African basil, efinrin	Lamiales	Lamiaceae	<i>Ocimum gratissimum</i> L.	Leaves	Ola et al., 2009
Cat's whiskers, orthosiphon	Lamiales	Lamiaceae	<i>Orthosiphon stamineus</i> Benth.	Leaves	Olah et al., 2003
Rabdosia	Lamiales	Lamiaceae	<i>Rabdosia rubescens</i>	Leaves	Tang et al., 2011
Mules foot fern	Marattiales	Marattiaceae	<i>Angiopteris lygodiiifolia</i>	Frond	Hasegawa and Taneyama, 1973
Lesser neptune grass	Najadales	Cymodoceaceae	<i>Cymodocea nodosa</i> (Ucria) Asch.	Leaves (fresh and detrital) and rhizomes	Grignon-Dubois and Rezzonico, 2013
Manatee grass	Najadales	Cymodoceaceae	<i>Syringodium filiforme</i> Kutz.	Leaves	Nuissier et al., 2010
Mediterranean tapeweed, neptune grass	Najadales	Posidoniaceae	<i>Posidonia oceanica</i> L. Delile	Shoots, leaves, and roots	Cariello and Zanetti, 1979; Cariello et al., 1979; Agostini et al., 1998; Dumay et al., 2004; Haznedaroglu and Zeybek, 2007; Heglmeier and Zidorn, 2010
Eelgrass, seawrack	Najadales	Zosteraceae	<i>Zostera marina</i> L.	Leaves	Pilavtepe et al., 2012
Japanese deer fern	Polypodiales	Blechnaceae	<i>Struthiopteris niponica</i> , <i>Blechnum nipponicum</i>	Frond	Hasegawa and Taneyama, 1973
Tree fern	Polypodiales	Cyatheaceae	<i>Cyathea fauriei</i>	Frond	Hasegawa and Taneyama, 1973
Squirrel's foot fern	Polypodiales	Davalliaceae	<i>Davallia mariesii</i>	Frond	Hasegawa and Taneyama, 1973
Western brackenfern	Polypodiales	Dennstaedtiaceae	<i>Pteridium aquilinum</i> (L.) Kuhn	Frond	Hasegawa and Taneyama, 1973
Japanese painted fern	Polypodiales	Dryopteridaceae	<i>Athyrium niponicum</i>	Frond	Hasegawa and Taneyama, 1973
Bladder fern	Polypodiales	Dryopteridaceae	<i>Cystopteris japonica</i>	Frond	Hasegawa and Taneyama, 1973
Wood fern	Polypodiales	Dryopteridaceae	<i>Dryopteris africana</i>	Frond	Hasegawa and Taneyama, 1973
Champion's wood fern	Polypodiales	Dryopteridaceae	<i>Dryopteris championii</i>	Frond	Hasegawa and Taneyama, 1973

(Continued)

Table 1 | Continued

Common name(s)	Order	Family	Genus and species	Plant parts evaluated and chicoric acid found in	References
Autumn fern, Japanese wood fern, Copper shield fern	Polypodiales	Dryopteridaceae	<i>Dryopteris erythrosora</i>	Frond	Hasegawa and Taneyama, 1973
Sensitive fern	Polypodiales	Dryopteridaceae	<i>Onoclea sensibilis</i> L.	Frond	Hasegawa and Taneyama, 1973
Trifid holly fern	Polypodiales	Dryopteridaceae	<i>Polystichum tripterum</i> , <i>Aspidium tripterum</i> , <i>Dryopteris triptera</i>	Frond	Hasegawa and Taneyama, 1973
Manchurian fern	Polypodiales	Dryopteridaceae	<i>Woodsia manchuriensis</i>	Frond	Hasegawa and Taneyama, 1973
Forked fern	Polypodiales	Gleicheniaceae	<i>Gleichenia japonica</i>	Frond	Hasegawa and Taneyama, 1973
Japanese climbing fern	Polypodiales	Lygodiaceae	<i>Lygodium japonicum</i> (Thunb.) Sw.	Frond	Hasegawa and Taneyama, 1973
Royal fern	Polypodiales	Osmundaceae	<i>Osmunda regalis</i> L.	Frond	Hasegawa and Taneyama, 1973
Polypody	Polypodiales	Polypodiaceae	<i>Neocheiropteris ensata</i> , <i>Polypodium ensatum</i> , <i>Neolepisorus ensatus</i> , and numerous other synonyms.	Frond	Hasegawa and Taneyama, 1973
Maidenhair fern	Polypodiales	Pteridaceae	<i>Adiantum monochlamys</i>	Frond	Hasegawa and Taneyama, 1973
Carrot fern, Japanese claw fern, claw fern	Polypodiales	Pteridaceae	<i>Onychium japonicum</i>	Frond	Hasegawa and Taneyama, 1973
Cretan brake	Polypodiales	Pteridaceae	<i>Pteris cretica</i> L.	Frond	Hasegawa and Taneyama, 1973
Chinese firethorn	Rosales	Rosaceae	<i>Pyracantha fortuneana</i> Li	Fruit	Zhao et al., 2013

They are alphabetically listed by order, family, genus, and species (USDA-NRCS, 2013; and reference listed in table). Common names and the plant fractions used in the referenced research papers are also summarized. Multiple genus and species listed within a cell are hybrids or synonyms.

acid) but also quantitative analyses. For example, Perry et al. (2001) showed >50% loss of chicoric acid when water was substituted for ethanol as the extraction solvent. Sample harvest timing (growth stage/development), handling, preparation, hydrolysis, and purification steps for optimum phenolic retention are areas to carefully consider when undertaking a detailed analysis.

We (Lee and Scagel, 2009, 2010; Lee, 2010; Scagel and Lee, 2012) were the first to discover that the second principal phenolic in basil (*Ocimum basilicum* L.) leaves was chicoric acid. Our suspicion is that identification of this basil compound was overlooked for so long was due to chicoric acid's prompt and rapid degradation during extraction procedures (Perry et al., 2001; Lee and Scagel, 2009). Lack of commercially accessible standard, in the past, also contributed to the delay in identification in basil (Lee and Scagel, 2009). In addition to extraction and analysis technical differences, a variety of sample preparation conditions contributed toward the disparity of reported chicoric acid levels in the literature. We demonstrated that blanching was a straightforward initial sample extraction step critical for high retention of phenolic compounds in samples high in native enzymes (Lee

et al., 2002; Lee and Scagel, 2009; Zhang et al., 2011). Optimal extraction procedures for chicoric acid may differ from other compounds that may be of interest in plant samples. Chicoric acid extraction performance differed from alkamide extraction in *E. purpurea* roots and shoots (Stuart and Wills, 2000). This work also demonstrated other critical considerations for optimizing extraction procedures including the influence of particle size (smaller particles of root material permitted greater chicoric acid extraction), solid/solute to extraction solvent ratio (1 part solid to 8 parts solvent), extraction temperature (60°C), and ethanol solvent to water ratio (60% ethanol: 40% water). The ideal extraction condition for maximum alkamide was different than that for chicoric acid (90% ethanol: 10% water at 20°C; Stuart and Wills, 2000).

Different solvent systems for extraction have been evaluated and efficiency of extraction can vary between phenolics because these compounds can vary in their polarity and accessibility. Extraction of chicoric and chlorogenic acids in *Rauvolfia serpentina* (L.) Benth. Ex Kurz shoots and roots was greatest in aqueous acetonitrile > acidified (HCl) acetonitrile > aqueous

methanol > aqueous ethanol > acidified (HCl) methanol > acidified (HCl) ethanol > 60°C water > ambient temperature water (Nair et al., 2013). In contrast, acidified (HCl) acetonitrile was considered a more superior extracting solution for caftaric and caffeic acids, and aqueous methanol for quercetin-rutinoside (a flavonol). Chicoric acid in *E. purpurea* samples was found to degrade or conjugate under conditions that inhibit degradation of caffeic acid derivatives (Nüsslein et al., 2000).

Current routine sample extraction procedures for chicoric acid are time consuming and labor intensive, and there has been little research on optimizing extraction for specific plants, plant structures, and multiple target compounds. Sample processing procedures in the future that allow for rapid sampling of a large number of samples for chicoric acid analyses are needed improve the quality and reliability of research results from greenhouse and field experiments.

BIOSYNTHESIS

The biosynthetic pathway of L-chicoric acid (the most abundant form) is still not well known; although it is generally understood to form via the shikimic acid/phenylpropanoid pathway as other phenolic acids, analogous to the conjugation of caffeic acid derivatives of rosmarinic acid or chlorogenic acid (Kuhn et al., 1987; Shetty, 2001; Petersen and Simmonds, 2003; Petersen et al., 2009). Overviews of phenolic biosynthetic pathway were well reviewed by Dixon and Paiva (1995); Vogt (2010), and Cheynier et al. (2013). To date, we found no published research on the specific enzymes involved in L-chicoric acid biosynthesis. While it is possible that some *meso*-chicoric acid findings may have occurred from isomerization of L-chicoric acid in methanol extracts prior to compound separation by HPLC, there have been some efforts in identifying the enzymes (e.g., hydroxycinnamoyltransferases) involved in *meso*-chicoric acid biosynthesis (Hohlfeld et al., 1996). Techniques using hairy root cultures (Liu et al., 2012) and molecular analyses (Baum et al., 2001; Rana and Chandra, 2006) may help expand our knowledge of chicoric acid biosynthesis in the future.

PLANT KINGDOM AND WITHIN PLANT DISTRIBUTION

VARIATION AMONG PLANT TAXA

To date, chicoric acid has been found in plants of at least 13 orders, 25 families, and 63 genera and species (listed in Table 1). It has been most often reported in either the family Asteraceae (Aster family)—20 genera and species, or the family Dryopteridaceae (Wood fern family)—8 genera and species. Production of chicoric acid does not appear to be ubiquitous in taxon within a plant family or sub-family. Chicoric acid has been detected in leaves of many genera in the Asteraceae, however, some genera within this family may not produce chicoric acid in leaves (e.g., *Achillea*, *Arnica*, *Cnicus*, *Echinops*, *Inula*, *Petasites*, *Solidago*, and *Tanacetum*; Jaiswal et al., 2011). Two genera within the Cichorioideae and Asteroideae sub-families of the Asteraceae are well-known for their chicoric acid production (e.g., *Cichorium*, *Echinacea*) yet chicoric acid has not been detected in other genera within these plant sub-families (8 genera listed above; Jaiswal et al., 2011).

Chicoric acid production varies within genera and within species. Within the genus *Echinacea* there is a wide range of chicoric acid production (Perry et al., 2001; Binns et al., 2002; Pellati et al., 2005; Sabra et al., 2012), with *E. purpurea* containing the greatest concentration of the nine species (Table 1). So, it is not surprising that among three popular and widespread *Echinacea* dietary supplements, preparations of *E. purpurea* contained higher levels of chicoric acid than either *E. pallida* or *E. angustifolia* (Perry et al., 2001; Binns et al., 2002; Pellati et al., 2005). Chicoric acid can also vary greatly between cultivars; Kwee and Niemeyer (2011) examined 15 basil cultivars and reported a chicoric acid range from 3 to 278 mg 100 g⁻¹ dry weight (93 fold difference; and Ribas-Agusti et al. (2011) recorded chicoric acid concentrations from 23 to 1388 mg 100 g⁻¹ fresh weight when they evaluated 13 cultivars of romaine lettuce.

VARIATION AMONG PLANT STRUCTURES

There is some confusion surrounding what part of the plant is suitable for use in herbal medicine particularly in the US. The European Medicine Agency (London, England) maintains a list of the plant fractions approved for human herbal usage. For example, the European Medicine Agency lists the roots of *E. angustifolia* DC. and *E. pallida* Nutt. as recognized for herbal medicine, and the whole plant (aerial and roots) of *E. purpurea* based on traditional uses and scientific data. Chicoric acid can vary widely with plant age and portion. For example, in roots and shoots of *E. purpurea* grown ranged from 203 to 3855 mg 100 g⁻¹ dry weight, an over 18 fold difference in both young and mature plant fractions (Qu et al., 2005).

Phenolic distribution within a plant are known to vary. We (Lee and Scagel, 2010) reported chicoric acid allocation of flowering *E. purpurea*, measuring over a 4 fold difference from sections highest to those lowest: leaves > roots > flowers > stems; 93–391 mg 100 g⁻¹ fresh weight of fraction. Similar findings were reported by Molgaard et al. (2003) where chicoric acid in *E. purpurea* leaves > rootstock > flower head $\approx$ root > stem (4.5 fold difference; 930–4240 mg 100 g⁻¹ dry weight). In contrast, chicoric in different plant parts of *E. purpurea* were reported in the descending order: flowers > leaves > stems > roots (compared in dry weight; Lin et al., 2011). Similar discrepancies among plant structures in chicoric acid accumulation were reported for Neptune grass (*Posidonia oceanica* L. Delile) where no chicoric acid was detected in leaves (Dumay et al., 2004) but others found it in leaves (Cariello and Zanetti, 1979; Heglmeier and Zidorn, 2010). Conflicting reports of chicoric acid accumulation among plant structures have also been reported for *E. angustifolia* where chicoric acid has (Hu and Kitts, 2000; Zheng et al., 2006) and has not (Li and Wardle, 2001) been detected in roots, and has (Zheng et al., 2006) and has not (Sloley et al., 2001) been detected in leaves. Differences in plant age and growing environment among these studies may account for the differences in how much chicoric acid accumulated in different structures.

Plant development and growing condition may influence accumulation of chicoric acid within a plant structure. For example, green leafy parts of endives were found higher in chicoric acid than the etiolated leaves, and endive green veins were slightly higher than white veins (Goupy et al., 1990). Outer

leaves from lettuce (numerous cultivars and genotypes evaluated) were higher in chicoric acid than inner leaves (Winter and Herrmann, 1986; Hohl et al., 2001). Chicoric acid concentration coincided with color within a loose-leaf lettuce head, red > green > white, which matched from outer leaves, to inner leaves, and to midribs (Gil et al., 1998).

An uneven distribution of phenolics within plants is not surprising, as portions' susceptibility to biotic stresses naturally (e.g., defense or reproduction), results in supply dependent upon the plant's need (Snook et al., 1994; Dixon and Paiva, 1995; Harborne, 1999). Understanding what factors regulate expected chicoric acid allocation in plants could be useful to tailor the concentration of commercial products by including (or not including) certain plant fractions.

IMPACT ON CONSUMER PRODUCTS

Supplements and other consumer products have shown even greater variation in chicoric acid content than reported for plant samples. For example, Molgaard et al. (2003) measured a 389 fold difference (from not detectable to 389 mg 100 mL⁻¹; $n = 13$) of chicoric acid in *Echinacea* extracts, and a 3460 fold difference (from not detectable to 3460 mg 100 g⁻¹; $n = 6$) of chicoric acid in *Echinacea* capsules. A range of 410–2140 mg 100 g⁻¹ dry weight was reported for *E. purpurea* root and aerial samples ($n = 62$) purchased from herb traders (Wills and Stuart, 1999), and a range of 310–877 mg 100 g⁻¹ dry weight ($n = 24$ accessions) in fall dandelion flower heads (*Leontodon autumnalis* L.; Grass et al., 2006). The diverse chicoric acid concentrations found within the basil, lettuce, and *Echinacea* samples summarized above emphasize the caution necessary when investigating chicoric acid's possible health benefits, especially if the evaluation is from a single form/sample of these plant materials.

Conflicting reports of chicoric acid accumulation in certain plant taxon can impact the potential for certain products to provide consumers with chicoric acid. Some of the chicoric acid reports listed in **Table 1** have yet to be independently confirmed (e.g., peanut shoots, zucchini, or Chinese firethorn fruit). For example, Snook et al. (1994) reported chicoric acid concentration in nine varieties of peanut (*Arachis hypogaea* L.) leaf terminals, but a recent study (Sullivan and Foster, 2013) did not detect chicoric acid in rhizoma peanut (*Arachis glabrata* Benth.) leaves, though different species and fractions were examined. Only two studies have found chicoric acid found in fruit (Chinese firethorn, Zhao et al., 2013; zucchini, Iswaldi et al., 2013). Potential sources of chicoric acid for consumer products need to be validated and tools for quality monitoring for chicoric acid in crops and product manufacturing need to be developed.

PLANT × GROWING ENVIRONMENT INTERACTIONS

Plant responses that cause chicoric acid accumulation can vary among plant, plant fraction, and growing conditions. To date, there have not been comprehensive enough body of research in this area to draw broad scale inferences. Since the quality of any agricultural product begins in the field, applying the knowledge gained from research in this area is vital for product improvements and production efficiency for growers and processors alike. A general review of managing phenolics in crop

production is available (Treutter, 2010). This section below summarizes research how plant growing environment may specifically alter chicoric acid accumulation.

PLANT DEVELOPMENT AND GROWING SEASON

Plant development is known to alter accumulation of phenolic compounds. Some research has indicated that more mature tissue contains less chicoric acid than younger tissue. For example young, actively growing Neptune grass leaves contained more chicoric acid when compared to older leaves (Cariello and Zanetti, 1979; Agostini et al., 1998; Haznedaroglu and Zeybek, 2007).

Maximum accumulation of chicoric acid may also occur at a specific time in plant development. For example, *meso*-chicoric acid accumulation reversed in *E. arvense* during development of sporophytes, with a continual buildup until to peak concentration at ~130 days of growth after which followed a steady decrease to 220 days of growth, demonstrating that sporophyte development altered phenolic acid reserves (Hohlfeld et al., 1996). The effects of tissue age may also vary with plant taxon. For example, in sowthistle (*Sonchus olearaceus* L.), mature leaves contained higher levels of chicoric acid compared to close-to-the-base and young leaves (Ou et al., 2012).

For some plants, however, no consistent relationship was found between tissue age and chicoric acid production. For example, no consistent trend was seen in chicoric acid levels of outer loose portions of leaf lettuce, as they fluctuated during seven sampling periods, although no statistics were conducted (Romani et al., 2002). In contrast, phenolic acids detected by HPLC in 5 varieties of lettuce and one variety of endive cultivated in hydroponic solutions chicoric acid concentrations in changed substantially with time of year and plant age (Amimoto and Fukui, 1996).

Time of year can alter accumulation of phenolics beyond its effects on plant development. For example, *E. purpurea* and *E. pallida* roots contained higher levels of chicoric acid in late spring than in other development stages/season (early winter, early spring, summer, and mid autumn; Thomsen et al., 2012). An earlier study found that summer harvested *E. purpurea* samples had more chicoric acid than those harvested in the fall (Perry et al., 2001). In some studies, however, no consistent relationship was found between time of year and chicoric acid production. For example, different harvest times (winter vs. spring) did not alter chicoric acid among six lettuce cultivars that were grown in the greenhouse (Nicolle et al., 2004).

Separating out the effects of time of year and plant development on chicoric acid accumulation has been indirectly investigated. Red oak leaf lettuces were grown at different temperatures to evaluate the influence of cool cultivation on plant phenolic composition (Becker et al., 2013). Plants were grown in either at 10/15°C day/night (warm treatment) or 12/7°C day/night (cool treatments) for up to 52 days. Heads from cool-cultivated plants contained higher concentration so chicoric acid than warm-cultivated plants 26 days after planting; however, this was interpreted a developmental difference between plants, not the direct effect of temperature on chicoric acid production.

Much of the research on chicoric acid production has used lettuce and *Echinacea* species or cultivars. Lettuce is an annual plant

while *Echinacea* is a herbaceous perennial plant. Seasonal changes in composition in annual and perennial plants can differ substantially and their metabolism can be regulated by different intrinsic (genetic) and environmental triggers. Many of the seemingly conflicting reports concerning effects of tissue maturity and growing season on chicoric acid accumulation may be related to whether the plants being evaluated are annual or perennial in nature. Accumulation of phenolic acids in plants are the net end product of their anabolism and catabolism. The reason for anabolism and catabolism of chicoric acid during plant growth is unknown. For growers and producers, these fluctuations in chicoric acid levels with tissue maturation and throughout the growing season should be taken into consideration before scheduling harvest dates or planning herbal product manufacturing (Perry et al., 2001).

CULTIVATION

Domestication of crops, through its effects on growing environment and breeding/selection can alter plant composition. Agronomic production conditions may decrease chicoric acid production compared to plants growing in their natural environment. Chicoric acid disappeared in some *Echinacea* species after wild-collected plants were transplanted in a greenhouse (Binns et al., 2002). For example, *E. laevigata* roots accumulated chicoric acid after transplantation, but the opposite trend was observed in *E. pallida*, *E. paradoxa*, and *Echinacea* hybrids. In *E. sanguinea* Nutt., chicoric acid levels decreased in the roots as flower-head chicoric acid level increased during flower maturity. Wild *Echinacea* flower heads had more overall chicoric acid than cultivated heads (Binns et al., 2002).

Decreased accumulation of chicoric acid between wild plants and those grown in production systems may be simply related to greater stresses in the wild increasing phenolic accumulation (Dixon and Paiva, 1995). Similarly, environmental differences among production systems may alter plant stress responses, including production of chicoric acid. For example, chicoric acid concentrations in field grown lettuce were >2 times greater than plants grown in a polycarbonate greenhouse 16 days after planting (Romani et al., 2002). Other environmental differences between agronomic systems and the natural environment that are not directly related to plant stress may also play a role. For example, higher chicoric acid levels occurred with increased elevation in a small number ($n = 7$) of smooth hawksbeard (*Crepis capillaris* L. Wallr.) flower heads collected from altitudes of 180–1060 m (Zidorn et al., 2005). Although this trend was not observed in fall dandelion flower head samples ($n = 24$) that had been collected at elevations from 10 to 2480 m (Grass et al., 2006).

Many proponents of organic agriculture liken it to more natural growing conditions for plants. Organic production systems have been reported to have no effect or increase chicoric acid in certain crops. “Verde” zucchini (*Cucurbita pepo* L.) samples from a public marketplace contained chicoric acid only when fruit were identified as organically grown (Iswaldi et al., 2013). In contrast, growing green leaf lettuce plants grown in conventionally managed field plots had similar chicoric acid concentrations and dry weight as plants grown in 5 year old certified organic plots when plants were fertilized (Rajashekar et al., 2012). Interestingly, in

the same study, growing plants with organic fertilizer (composted cattle manure and alfalfa hay) decreased chicoric acid concentrations compared to plants grown without additional fertilizer and, plants grown with non-organic fertilizer in non-organic plots had similar chicoric acid concentrations and greater dry weight than plants grown without additional fertilizer. These results suggest the differences in chicoric acid accumulation reported maybe a function of nutritional stress. Research on industrial bioproduction of chicoric acid indirectly lends support to increased stress increasing chicoric acid accumulation. Chicoric acid production by adventitious roots of *E. purpurea* cultured for 50 days in bioreactors was almost 4 times greater than concentrations in roots from field-grown plants (Wu et al., 2007b). Adventitious root production is considered a stress related response in many plants and causes an up-regulation of phenolic metabolism. There is an obvious need for more research on how chicoric acid accumulation in plants is regulated and how cultivation and production strategies can be used to optimize accumulation in plants.

MICROORGANISMS

Interactions between plants and microorganisms can cause an astounding variety of effects on plant composition that may result in accumulation of phenolic compounds due to stress or altered plant vigor. Pathogens can have a negative impact on plant growth but may increase phenolic accumulation. For example pathogen attack (cucumber mosaic virus and phytoplasma-prokaryote) decreased chicoric acid levels in *E. purpurea* roots (Pellati et al., 2011). Foliar application of carboxymethyl chitin glucan (a fungal elicitor produced by *Penicillium*) increased production of chicoric acid in roots of *E. purpurea* (Hudec et al., 2007). Increased chicoric acid accumulation in response to pathogen infection or pathogen elicitors supports a link between plant stresses and enhanced chicoric acid production. The linkage between pathogen infection and chicoric acid production has received little research attention with plants.

Beneficial bacteria and fungi (including mycorrhizal fungi) can enhance plant growth but have been shown to have positive, negative, and no influence on accumulation on plant phenolic composition. For example, arbuscular mycorrhizal fungus (AMF) colonization did not alter chicoric acid levels in either “Genovese Italian” or “Purple Petra” basil leaves and stems (Lee and Scagel, 2009). In contrast the shoots of *E. purpurea* showed no change in chicoric acid levels due to AMF colonization, but the roots had more chicoric acid with colonization (Araim et al., 2009). Increased chicoric acid accumulation in roots of AMF colonized plants was hypothesized to be a result of the plants reaction to fungal infection. In contrast, the effects of AMF on chicoric acid production in “Cinnamon,” “Siam Queen,” “Sweet Dani,” and “Red Rubin” basil, revealed that both AMF and phosphorus (P) fertilizer rate increased chicoric acid accumulation in basil shoots (Scagel and Lee, 2012). These results suggest that neither nutritional stress or the plant AMF infection consistently increases chicoric acid production. Inoculation of crop plants with mycorrhizal fungi is becoming a more routine practice in production systems for several crops. Improved knowledge of how these fungi may alter secondary plant metabolism is needed to understand

whether these fungi can be used to manipulate target compounds such as chicoric acid.

LIGHT, TEMPERATURE, AND OTHER STRESS ELICITORS

The direct effects of selected environmental stresses (stress elicitors like heat, light, ultrasound, hormone, etc.) on chicoric acid accumulation have been investigated experimentally in very few crops. In most, but not all cases, increased levels of treatments that resulted in greater plant stress increased chicoric acid production.

Five-week old “Baronet” lettuce plants were subjected to brief simulated environmental stresses and sampled 1 day before and <1 day, 1 day, and 3 days after stress (Oh et al., 2009). A brief heat shock (40°C for 10 min at 90% relative humidity, RH) increased chicoric acid 3 days after treatment compared to control plants. Cold treatment (4°C for 1 day in a growth chamber) increased chicoric acid 1 h, 1 day, and 3 days after treatment. High light (800 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 1 day) exposure had no influence on chicoric acid 1 h after exposure, but increased concentrations 1 day and 3 days after exposure. On average the heat and cold stress treatments resulted in ~ 2 fold increase in chicoric acid while high light exposure increased concentrations by ~ 7 fold.

Reducing photosynthetic photon flux density (PPFD) from 410 $\mu\text{mol m}^{-2} \text{s}^{-1}$ to 225 $\mu\text{mol m}^{-2} \text{s}^{-1}$ had no influence on chicoric acid concentrations in red oak leaf lettuce (“Eventai”) even though lower light decrease plant dry weight and accumulation of reducing sugars in 4 week old plants (Becker et al., 2013).

Manipulation of the growing environment in bioproduction of chicoric acid has been investigated. Manipulating growth temperatures (10, 15, 25, 20, and 30°C) and photoperiods (24 h light/day to 24 h dark/day; 40 $\mu\text{mol m}^{-2} \text{s}^{-1}$ fluorescent light) in *E. purpurea* suspension cell cultures revealed that 20°C and 3 h light and 21 h dark for 5 weeks produced the most chicoric acid (Wu et al., 2007a). Chicoric acid still accumulated in *E. purpurea* cultures that were grown in complete darkness for 5 weeks. Unfortunately no statistics were performed on the results. Ultrasound treatments successfully were used to enhanced chicoric acid production in hairy root cultures of *E. purpurea* (Liu et al., 2012). In field and controlled environment production systems, use of known elicitors of plant stress metabolism have been evaluated for their effects on chicoric acid production. Foliar application of stress or defense elicitors (acetyl salicylic acid, ASA; salicylic acid, SA; and methylsalicylic acid, MSA) applied at 0, 10, 100, and 1000 μM concentrations to *Rauvolfia serpentina* increased chicoric acid production in shoots and roots of plants grown in a controlled environment conditions (Nair et al., 2013). In this study, the most dilute elicitor solutions had the greatest influence on chicoric acid in shoots while more concentrated elicitor solutions had the greatest effect on chicoric acid in roots. Of the three elicitors evaluated SA had the greatest influence on chicoric acid. In contrast, other phenolics (e.g., chlorogenic acid, caftaric acid) were more sensitive to MSA. A similar study evaluated the effects of foliar application of ASA, SA, MSA, and titanium (IV) ascorbate at different concentrations on chicoric acid in *E. purpurea* growing in the field for 2 years (Kuzel et al., 2009). In comparison to controls, chicoric acid concentrations in shoots were only greater in plants treated with SA at 10 μM and to a lesser extent titanium (IV) ascorbate.

All elicitors increased chicoric acid in roots compared to controls.

SALINITY AND NUTRIENTS

Nutrients and salinity are intimately linked in production of crops in field and controlled culture. A relatively large body of literature is available on the effects of plant nutrition on production and regulation of other several phenolic compounds see review by Treutter (2010). However, the direct effects of specific salinity rates (as assessed by electrical conductivity, EC) and selected nutrients on chicoric acid accumulation have not been investigated experimentally in many crops, and reported results vary. Some studies assessing how salinity and nutrients influence chicoric acid only report concentrations and not total content; therefore in these studies it is impossible to determine whether treatments altered production or the differences in chicoric acid are only a result of treatments on plant growth.

Sensitivity of chicoric acid production to salinity varies among species, with evaluated levels of salinity having negative, positive, and no effect of chicoric acid accumulation. Two species of *Echinacea* (*E. purpurea* and *E. pallida*) expressed chicoric acid concentrations differently in response to salinity (0, 50, 75, and 100 μM sodium chloride hydroponic solution) (Sabra et al., 2012). Chicoric acid concentrations in *E. purpurea* increased considerably with salt concentration up to 75 μM , but was lowest at the greatest (100 μM) salinity; while in *E. pallida* chicoric acid concentrations were elevated in both of the higher (75 and 100 μM) salinity treatments (Sabra et al., 2012). Salinity treatments (NaCl, 5 and 50 mol m^{-3}) decreased plant dry weight and chicoric acid in leaves but increased chicoric acid in roots of 4-month-old *E. angustifolia* grown in the field (Montanari et al., 2008).

In contrast to the results in the *Echinacea* study above, baby romaine lettuce showed no differences in chicoric acid concentration after growth in various salinities (2.8, 3.8, and 4.8 dS m^{-1}) and storage (4°C for 10 days; Chisari et al., 2010). In another study with hydroponically grown “Capitata” lettuce increasing salinity from 0 to 150 mM NaCl increased concentrations of total phenolics and the individual phenolic acids identified (including chicoric acid) after 10 days of salinity treatment (Garrido et al., 2014). Increased salinity increased concentration of phenolic acids by 22.4% but no data was presented for the individual phenolic acids; additionally, increased salinity decreased plant biomass therefore the salinity may not have altered the production of chicoric acid. In a study with 5 varieties of lettuce and one variety of endive cultivated in hydroponic solutions with 2.4 and 4.8 mS cm^{-1} , phenolic acids detected by HPLC indicated that there were no qualitative differences in phenolic acids at different EC; however, chicoric acid concentrations in lettuce was decreased at the highest EC (Amimoto and Fukui, 1996).

Sensitivity of chicoric acid production to various forms of fertilizer varies among species, with evaluated fertilizer forms having negative, positive, and no effect of chicoric acid accumulation. In some research nutrient application rate was also altered with fertilizer form; therefore the effects of fertilizer form on chicoric acid cannot be separated from the differences in nutrient application rates among treatments. Fertilizer additions (organic or

non-organic) decreased chicoric acid accumulation in green leaf lettuce ("Baronet") (Rajashekar et al., 2012). Use of a commercial form of cow manure vs. conventional fertilization did not result in chicoric acid differences within chicory leaves, in either water stressed plants or unstressed ones (Heimler et al., 2009). Nitrogen (N) source in fertilizer (nitrate vs. mix of ammonium and nitrate) had no influence on growth of 4-month old *E. angustifolia*, but plants grown with only nitrate had higher concentrations of chicoric acid in leaves and roots than plants grown with a mix of nitrate and ammonium (Montanari et al., 2008). Growing *E. pallida* and *E. purpurea* at 3 different fertilizer rates for 7 months in a field planting had no significant influence of fertilizer rate on root weight or chicoric acid concentration although root growth and chicoric acid concentration of roots tended to decrease with increasing fertility (Dufault et al., 2003).

Increased P rate enhanced chicoric acid production in basil and shoot accumulation of chicoric acid was correlated with enhanced uptake of P, Ca, Mg, B, Fe, and Mn (Scagel and Lee, 2012). Chicoric acid production in basil did not correlate with the uptake of K or Zn even though production of other rosmarinic acid (the main phenolic in basil) correlated with uptake of these nutrients (Scagel and Lee, 2012). In contrast, K treatment (0.005 M) increased chicoric acid in basil leaves after 30 days of post-germination growth (Nguyen et al., 2010).

POST-HARVEST HANDLING, PROCESSING, AND STORAGE

Optimizing processing steps to better extract or retain chicoric acid directly improves commercial product quality (Lee, 2010). Chicoric acid has been proposed as a quality control indicator compound due to its rapid degradation, in contrast to other secondary metabolites, within plant materials (Stuart and Wills, 2003; Lee, 2010). This section summarizes how different post-harvest and processing conditions influence chicoric acid retention.

POST-HARVEST REACTION OF PLANT TISSUES

Harvesting procedures that purposefully wound the plant can elicit stress responses from plants that may enhance accumulation of phenolic compounds, including chicoric acid. Chicoric acid levels of iceberg lettuce midrib increased from harvest until day 2 of storage at 7°C (Luna et al., 2012). Iceberg lettuce butt (cut stem/midrib end after harvesting) started to produce chicoric acid 48 h after the initial wounding, and showed biosynthesis highest at the wound site, then progressively decreasing up the stem away from the wound site, to eventually being undetectable (Tomas-Barberan et al., 1997). They also showed that applying a calcium solution (0.30 M calcium chloride) to the wound site inhibited browning (visually undesirable trait) on the stem surface while retaining chicoric acid. These results suggest that chicoric acid production is linked to wound response.

In contrast wounding the white, green, and red tissues of "Lollo Rosso" lettuce increased chlorogenic and caffeoyltartaric acids but had no influence on chicoric acid (Ferrerres et al., 1997). Differences in wound-induced phenolic responses among lettuce varieties or tissues may be a function of the pre-existing abundance of phenolics prior to wounding and different levels of native enzymes. Tissues or varieties with an abundance of

phenolics were hypothesized to have a lower or less detectable wound-induced response in phenolics.

WASHING AND RINSING

Post-harvesting procedures used to clean material and decrease potential contamination as part of the processing chain (washing and rinsing) can alter the phenolic composition of plant tissues. Of the few studies done in this area of research, mostly on lettuce, washing or rinsing plant material after harvest has little effect on chicoric acid retention. There was no treatment differences in chicoric acid levels in rinsed (control, ozonated water, ozonated with UV light treatment, and chlorinated water) shredded iceberg lettuce after air storage (Beltran et al., 2005). There were minimal changes (or differences) in chicoric acid among iceberg lettuce samples after a washing regime (a number of different preparations; cut then rinsed, rinsed then cut, chlorine free tap water, 100 ppm chlorinated water, or ozonated water, etc.) (Baur et al., 2004). Shredded lettuce chicoric acid levels did not alter between washing treatments (tap water rinsed, distilled water dipped, and 100 ppm chlorinated water dipped), but as this study made no measurement of pre-shredded lettuce, loss or retention cannot be determined (Kenny and O'Beirne, 2009).

STORAGE

Post-harvesting storage, prolong availability of plant tissues over time, can alter the phenolic composition of plants tissues. For lettuce, storage conditions for plant material after harvest decreases, increases, or has little effect on chicoric acid retention. Concentrations of chicoric acid in shredded leaves of iceberg lettuce decreased after modified atmosphere storage (mixture of oxygen and carbon dioxide) of 13 days at 4°C (Beltran et al., 2005). There were minimal changes (or differences) in chicoric acid among iceberg lettuce samples after 9 days of dark storage at 4°C (Baur et al., 2004). Five of the six lettuce cultivars evaluated after 7 days of 5°C storage increased in chicoric acid content (Cantos et al., 2001), indicating chicoric acid can be biosynthesized in lettuce tissue during post-harvest storage. After 24 h storage at 4°C, chicoric acid in fresh cut leaves of lettuce purchased from local marketplaces increased (~2 fold), then decreased to the original level after 72 h storage (Degl'Innocenti et al., 2008). In contrast, chicoric acid concentration increased in fresh cut endive leaves after 24 h storage and remained elevated by 72 h.

Modified atmosphere packaging (MAP; air replaced with 3% oxygen, 8% carbon dioxide, and 89% nitrogen) did not enhance chicoric acid retention from that of air in the red and green parts of the lettuce, though white lettuce tissues increased in chicoric acid with the MAP (Gil et al., 1998). MAP of fresh-cut romaine lettuce stored for 10 days reportedly increased chicoric acid in leaves and light exposure of package product had no influence on chicoric acid; however, only total phenolic content was presented (Martínez-Sánchez et al., 2011).

Similar disparate results have been reported for the effects of storage on *E. purpurea*. Lowered moisture/humidity during *E. purpurea* post-harvest storage increased chicoric acid retention of final products (Kim et al., 2000; Wills and Stuart, 2000). Storage of freeze dried *E. purpurea* at 10 and 20°C in polyethylene

terephthalate/aluminum boil/polyethylene bags had no influence on chicoric acid during 180 days (Lin et al., 2011). Storage at 30°C decreased chicoric acid after 90 days. In the same study, storage of freeze dried *E. purpurea* at 40 and 60% RH in the dark did not influence chicoric acid during 180 days. Storage at 80% RH decreased chicoric acid after 135 days. Additionally, dark storage of freeze dried *E. purpurea* at 40% RH in nylon/polyethylene bags did not influence chicoric acid during 180 days. Light decreased chicoric acid production after 90 days.

DRYING

Drying is a commonly used food preservation method. Drying is used to preserve phenolics and other compounds (essential oils, etc.) in plant materials; however, the effects of drying will vary depending on the drying procedure, the type of plant material, and chemical in question (Lee and Scagel, 2009; Lee, 2010).

Freeze drying, though gentler than ordinary air drying, decreased phenolics in basil preparations by 13% compared to fresh material (Lee, 2010). Freeze drying of *E. purpurea* flowers retained chicoric acid better than vacuum drying, microwave drying, or air drying (Kim et al., 2000). Freeze dried flower heads also retained more color and had less browning (Kim et al., 2000). Drying *E. purpurea* for different lengths of time using vacuum freeze drying, cool air drying, and hot air drying revealed that vacuum freeze drying generally retained higher concentrations of chicoric acid and cool air drying greater than hot air drying (Lin et al., 2011). Others have demonstrated that microwave drying of *E. purpurea* retained more chicoric acid than steam heating, hot water bath, or air-drying (in decreasing order of chicoric acid retention; Zhang et al., 2011). Drying temperature can also play a role in retention of phenolics in plant materials. Higher air-drying temperature (70°C compared to 40 and 25°C) accelerated the loss of chicoric acid in *E. purpurea* flowers (Kim et al., 2000).

Echinacea purpurea aerial portions had a greater loss of chicoric acid than did roots during drying (Stuart and Wills, 2003). Different drying temperatures did not degrade *E. purpurea* alkamide in a manner like chicoric acid (Stuart and Wills, 2003), indicating the importance of monitoring all target compounds until their traits are fully understood. Over-drying had no effect on chicoric acid retention (Stuart and Wills, 2003).

ADDITIONAL MINIMAL PROCESSING AND ADDITIVES

There are many other processing chain procedures that have the potential to influence phenolic composition of plant materials or products because of their use of heat, additives, preservatives, etc. that may alter phenolic stability. There have been few studies done in this area of research, and most studies, to date, have used *E. purpurea*. High hydrostatic pressure (HHP) pasteurization to increase product microbial stability did not alter chicoric acid retention in *E. purpurea* flowers and roots over to unpasteurized samples (Chen et al., 2010). Additives like citric acid, malic acid, and dried Hibiscus (*Hibiscus sabdariffa* L.) flower glycerin extract increased the retention of chicoric acid (and other bioactives) in *E. purpurea* extracts (in glycerin) for 4 months at 25°C (Bergeron et al., 2002). Additives, including 0.05 M ascorbic acid, 0.10 M ascorbic acid, 30% ethanol, and 40% ethanol, helped *E. purpurea*

extracts to maintain a consistent chicoric acid concentration for 4 weeks (Nüsslein et al., 2000).

COOKING

The consumer method for use of plants and plant products that may have the greatest potential to alter their phenolic composition is cooking. Cooking can be used by the consumer on fresh materials as prior to consumption or storage. Boiling plant materials prior to consumption can increase, decrease, or have little effect on chicoric acid in chicory. Little chicoric acid was lost after boiling chicory leaves for 30 min. (Innocenti et al., 2005). Boiling stems for 8 min decreased the concentration of total phenolics in stems of “Galatina” chicory, but had no effect on concentration of total phenolics in stems of “Molfettese” chicory (Renna et al., 2014). In contrast, microwaving stems of both chicory varieties in water for 3 min increased concentration of total phenolics in stems (Renna et al., 2014). These authors hypothesized that these changes in total phenolics may reflect how the cooking techniques evaluated affected chicoric acid, the primary phenolic constituent of total phenolics in chicory (Innocenti et al., 2005).

Blanching harvested basil prior to freezing preserved chicoric acid levels better than basil frozen without an initial blanching step (Lee, 2010). Four *Ocimum* species total phenolics were compared after five cooking methods (blanching, boiling, steaming, sautéing, and high temperature via pressure-cooking) to control-fresh, though chicoric acid was not separately identified (Trakoontivakorn et al., 2012). HPLC profiles showed that pressure-cooked basil had the greatest loss of phenolics (Trakoontivakorn et al., 2012).

Additional work on chicoric acid levels remaining in the edible portions after cooking is needed. The lack of literature in this area is not surprising since most of the edible plant parts listed in **Table 1** are typically eaten raw or minimally processed (ready-to-eat salad mixes), as a tea, as pre-prepared herbal liquid extracts (alcohol free, contains alcohol, etc.), or as capsules.

BIOLOGICAL ACTIVITIES OF CHICORIC ACID

Chicoric acid properties have been reported to include anti-cancer, anti-obesity, antiviral, and anti-diabetic (King and Robinson, 1998; Pluymers et al., 2000; Charvat et al., 2006; Queffelec et al., 2008; Tusch et al., 2008; Tsai et al., 2012; Azay-Milhau et al., 2013; Xiao et al., 2013). For example, chicoric acid and its analogs have been claimed to possess anti-Human Immunodeficiency Virus (HIV) activity due to its involvement in HIV integrase inhibition, which could perhaps hinder HIV strain replication (King and Robinson, 1998; Charvat et al., 2006; Queffelec et al., 2008). Bel-Rhliid et al. (2012) reported a means of chicoric acid hydrolysis, facilitated by the probiotic bacterium (*Lactobacillus johnsonii*) after ingestion, but prior to absorption and metabolism. Due to the concerns surrounding all *in vitro* antioxidant measurements, chicoric acid antioxidant studies will not be summarized here, but the issues and research needed in this area have been well summarized elsewhere (Verhagen et al., 2010; Chiva-Blanch and Visioli, 2012).

Care should be taken when examining biological activities of a whole plant, due to the many bioactive components it likely has. For example, in *E. purpurea*, chicoric acid is only one of

the several compounds (alkamides-also known as alkylamides, other caffeic acid derivatives, polysaccharides, and glycoprotein) associated with purported health benefits of *E. purpurea* supplements (Barnes et al., 2005). But, a specific genus and species reported to contain a high concentration of chicoric acid does not guarantee that other bioactive compounds are high in the plant as well. For example, chicoric acid was found highest in wild grown old inflorescences of *E. sanguinea*, while alkamides were highest in roots of greenhouse cultivated *E. purpurea* originally collected from the wild (Binns et al., 2002). It should be noted that the immunostimulating properties of *Echinacea* products are still under investigation, and the evidence to date indicate its bioactivities were no better than placebo (Barnes et al., 2005; Gertsch et al., 2011; and references there in).

Consumer education and awareness in healthy eating has resulted in a surge in dietary phenolic consumption and market demand for these products. Although this review is only focused on the phenolic compound- chicoric acid, any health benefits of plants mentioned in this review require more data. For those wishing to supplement chicoric acid intake, it is still unclear which delivery form (food, pill, capsules, extract, etc.), or what level would be most effective for improving human health that minimizes possible risks of toxicity, herb/drug interactions, or synergistic and/or antagonistic effects. Despite excitement about chicoric acid's potential from antiviral researchers, or individuals and families affected by HIV, additional research is needed to understand the benefits of chicoric acid and its analogs.

CONCLUDING REMARKS

There is a strong need for a well-defined dietary supplement regulation in the US. Until dietary supplements receive the scrutiny as foods in the US, with minimum quantities of active ingredients, full ingredient disclosures, safety guidelines, sound science supporting claims, etc. the safest source of consuming chicoric acid is via food. Good review articles (Dufault et al., 2000; Cardellina II, 2002; Sanzini et al., 2011; Khan and Smille, 2012; Applequist and Miller, 2013) regarding these concerns and possible ways to improve our current US dietary supplement situation are available. Effective research and commercial use of plant derived chemicals requires well defined and validated chemical analyses (Lee, 2014). Methods for chicoric acid detection and quantification have improved substantially over the last 25 years; however, future work is needed to clarify the biosynthesis pathway of chicoric acid and the role of chicoric acid in the plant. Additionally, potential consumer benefits from chicoric acid can only be effectively realized after we have improved knowledge of growing and processing conditions for maximum retention, and how effects of domestic cooking may alter compound activity. As a supplement, there is a great deal of room for improvement in our knowledge of chicoric acid.

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Alkaline ionic liquids applied in supported ionic liquid catalyst for selective hydrogenation of citral to citronellal

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The challenge in preparation of ionic liquids containing a strong alkaline anion is to identify a suitable cation which can tolerate the harsh conditions induced by the anion. In this study, a commercial quaternary ammonium compound (quat) benzalkonium [ADBA] (alkyldimethylbenzylammonium) was used as a cation in the synthesis of different alkaline ionic liquids. In fact, the precursor, benzalkonium chloride, is a mixture of alkyldimethylbenzylammonium chlorides of various alkyl chain lengths and is commonly used in the formulation of various antiseptic products. The prepared ionic liquids were utilized as Supported Ionic Liquid Catalysts (SILCAs). Typically, a SILCA contains metal nanoparticles, enzymes, or metal complexes in an ionic liquid layer which is immobilized on a solid carrier material such as an active carbon cloth (ACC). The catalysts were applied in the selective hydrogenation of citral to citronellal which is an important perfumery chemical. Interestingly, 70% molar yield toward citronellal was achieved over a catalyst containing the alkaline ionic liquid benzalkonium methoxide.

Keywords: catalysis, hydrogenation, ionic liquids, supported ionic liquid catalysts, fine chemicals

INTRODUCTION

Ionic liquids have great potential for developing clean catalytic technologies. Ionic liquids can make a significant positive environmental impact as a replacement for volatile organic solvents. Volatile organic compounds are a major source of environmental pollution. Generally, ionic liquids are molten salts that possess a low melting point and negligible vapor pressure (Bonhôte et al., 1996; Rogers and Seddon, 2003; Deetlefs and Seddon, 2006). The two most applied, classical methods for the preparation of ionic liquids are the metathesis procedure and acid-base neutralization reaction. Metathesis implies a reaction of, e.g., a halide salt with a group 1 metal or ammonium salt of the desired anion (Welton, 1996).

Ionic liquids can be divided into acidic, neutral, or alkaline/basic ionic liquids. Acidic and alkaline ionic liquids represent new classes of acids and bases. Various ionic liquid anions can be classified as alkaline. These alkaline anions include e.g., formate, acetate, hydroxide, methoxide, butoxide, and the dicyanamide anions. With alkaline ionic liquids, the cation must be stable enough to tolerate the conditions emerging due to the alkaline anion. With alkaline ionic liquids, electrostatic interactions between the cation and anion are stronger than with neutral ionic liquids. The acidity or alkalinity of ionic liquids is governed by the strength of the cation or the anion (Hajipour and Rafiee, 2009).

Citral and its hydrogenation products such as citronellal are widely used in the perfumery and fine chemical industry. Ionic liquids can boost the activity of the supported catalyst in the hydrogenation of alkenes, citral and other α , β -unsaturated aldehydes (Mehnert et al., 2002; Virtanen et al., 2007). Ionic liquids can also stabilize metal nanoparticles and suppress aggregation resulting in enhanced catalytic activities. Citral hydrogenation

reactions with palladium catalysts and alkaline promoters such as potassium hydroxide have earlier demonstrated high selectivity toward citronellal (Salminen et al., 2012). It is proposed that the benzalkonium based ionic liquids could take the role of an alkaline promoter, like in the sample case of acetylation reactions of sugars and alcohols with dicyanamide based alkaline ionic liquids (Forsyth et al., 2002). Strong alkaline ionic liquids clearly enhanced the activity of Supported Ionic Liquid Catalysts (SILCA) in the hydrogenation of citral as well as the selectivity toward citronellal. It was also found out that alkaline ionic liquids can take the role of alkaline-modifiers, which was the case when e.g., potassium hydroxide was applied as a promoter leading to a much higher reaction rates and selectivities toward citronellal (Salminen et al., 2012).

MATERIALS AND METHODS

SYNTHESIS OF IONIC LIQUIDS

Ionic liquids were prepared by the metathesis procedure as follows: benzalkonium chloride ([ADBA][Cl]) (Acros Organics, 95%) and sodium methoxide (Aldrich, 95%) or potassium tert-butoxide (Acros Organics, 98%), in a molar ratio of 1:1, were dissolved in dichloromethane. The structures of these water miscible ionic liquids are illustrated in **Figure 1**. It should be noted that neither sodium methoxide nor potassium tert-butoxide are fully soluble in dichloromethane. The solution was stirred at room temperature for 24 h. The precipitated salt and unreacted starting material were filtered off. The ionic liquids dissolved in dichloromethane were washed with a small amount of water. The solvent (dichloromethane) was evaporated under reduced pressure and at an elevated temperature using a rotary evaporator. Consequently, the synthesized alkaline ionic liquids were

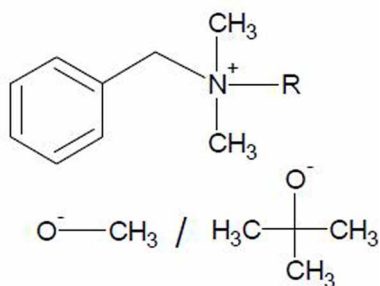


FIGURE 1 | Synthesized alkaline ionic liquids, benzalkonium methoxide ([ADBA][MeO]) and benzalkonium tert-butoxide ([ADBA][tBuO]). Alkyl distribution from C₈H₁₇ to C₁₆H₃₃.

investigated by means of thermogravimetric analysis (TGA), differential scanning calorimetry (DSC) and NMR-spectroscopy and applied in the preparation of SILCA.

CHARACTERIZATION OF IONIC LIQUIDS

The alkaline ionic liquids were evaluated for their mutual solubility in different solvents. DSC was applied for analyzing melting points and glass transition temperatures. The decomposition temperatures were analyzed by means of TGA. NMR spectra of the ionic liquids were analyzed applying Bruker AV400 instrument.

CATALYST PREPARATION

A simple straightforward catalyst preparation method was used (Mikkola et al., 2006). Catalysts were prepared as follows: approximately 150 mg of ionic liquid and 50 mg of palladium acetylacetonate (Aldrich, 99%) were both dissolved into acetone (Merck, p.a). The solutions were mixed together and then poured over a pre-dried active carbon cloth (ACC) Kynol® (approximately 1.2 g). The active carbon cloth containing the precursor and the ionic liquid was dried at 80°C 2 h. Both modified- and unmodified (traditional heterogeneous catalyst consisting of palladium nanoparticles on ACC) SILCAs were pre-reduced prior to the hydrogenation experiments. The catalysts were pretreated in a high-pressure semi-batch reactor (Parr Inc.). Pretreating was performed at 120°C under hydrogen flow of 10 bar for 1 h. Consequently, the catalyst containing palladium nanoparticles in an ionic liquid immobilized on ACC was obtained upon decomposition and reduction of the organic metal salt.

CATALYST CHARACTERIZATION

Catalysts containing alkaline ionic liquids were analyzed by means of energy-filtered transmission electron spectroscopy (EFTEM LEO 912 OMEGA) equipped with an energy dispersive X-ray detector. Prior to the characterization, the samples were crushed and dispersed in n-hexane or in water for the EFTEM analyses. Finally, the samples were dispensed on a specimen holder copper grid (e.g., carbon). The catalysts were analyzed as fresh and as spent ones.

Nitrogen physisorption (Carlo-Erba instruments, sorptometer 1900) was applied to determine the specific surface area and

pore volume of the materials. Dubinin's equation was applied to calculate the specific surface area whereas Dollim.-Heal method was used to calculate the micropore volume of the catalysts. Approximately 0.15 g of catalyst (active carbon cloth) was out-gassed for 3 h at 120°C to eliminate the moisture. Nitrogen was absorbed and desorbed from the sample material which was subjected to −196°C. The leaching of ionic liquids has been studied in our earlier work and it was discovered that since these ionic liquids are not mutually soluble within hexane, they don't leach from the support to the bulk solvent (Virtanen et al., 2007). The spent catalysts were extracted using methanol to study the accumulation of different hydrogenation decomposition products on the catalyst surface. The extractant solution was analyzed by means of gas chromatography as shown in the case of the citral hydrogenation samples.

CITRAL HYDROGENATION

The SILCA were applied in the hydrogenation of citral (Aldrich, 95%). Experiments were performed in a semi-batch reactor (Parr Instrument Company). The liquid volume of the reaction solvent was 250 ml. The temperature and stirring rate were controlled by a Parr 4843 control unit (Watlow control series 982). The stirring rate was adjusted to 1200 rpm in order to ensure operations under kinetic regime as confirmed by our earlier studies (Virtanen et al., 2007). All experiments were performed at a constant hydrogen pressure and temperature. Approximately 3 g of citral (0.02 mol) was dissolved in 250 ml n-hexane (Merck, >99%).

The samples from the reactor were analyzed by means of gas chromatography (Hewlett Packard 6890 GC with FID). Five hundred microliter of internal standard (0.02 M cyclohexanone in cyclohexane) was added into a 500 µl of sample. The GC column used was an Agilent DB-5 with a length of 60 m, inner diameter of 0.32 mm and a film thickness of 1 µm. The following temperature program was applied: 10 min at 100°C, then raised 5°C/min to 160°C. Temperature was then held 10 min at 160°C. At the end the temperature was increased 13°C/min to 200°C and kept constant for 1 min.

RESULTS AND DISCUSSION

CHARACTERIZATION OF IONIC LIQUIDS

The alkaline ionic liquids were tested for mutual solubility in methanol, acetone, and hexane. The ionic liquids were insoluble (within measuring accuracy) to hexane and soluble to acetone and methanol. The chlorine content of alkaline ionic liquids could not be determined by using AgNO₃ method because the ionic liquids reacted with AgNO₃. This was noticed when the same alkaline ionic liquids were synthesized from benzalkonium nitrate and the AgNO₃ test was positive. As the next step, DSC, a technique for analyzing melting points and glass transition temperatures, was applied. No melting points were found by using DSC (apparatus limit −100°C). The glass transition temperatures were −46°C for [ADBA][tBuO] and −45°C for [ADBA][MeO] salts, respectively. The color of ionic liquids was yellowish/light brown, which implies that some impurities were still present. The decomposition temperatures were analyzed by means of TGA. The decomposition temperatures were 160°C for [ADBA][MeO] and 140°C for [ADBA][tBuO], respectively.

The ^1H (400 MHz) and ^{13}C (400 MHz) NMR spectra of the ionic liquids were analyzed in deuterated chloroform (CDCl_3). The instrument used was a Bruker AV400. New peaks for the corresponding anion were observed in ^{13}C spectrum. ^1H NMR and ^{13}C NMR peaks for $[\text{ADBA}][\text{tBuO}]$. ^1H NMR (CDCl_3): δ 7.581, 7.334, 4.908 (s, 2H), 3.382 (m, 2H), 3.181 (s, 6H), 1.699 (s, 2H), 1.165 (m, 26H), 0.784 (t, 3H). ^{13}C NMR (CDCl_3): δ 133.17, 130.45, 129.01, 127.58, 84.10, 73.74, 68.77, 67.34, 63.52, 53.42, 49.56, 31.16, 29.2, 28.45, 26.25, 22.56, 14.00. ^1H NMR and ^{13}C NMR peaks for $[\text{ADBA}][\text{MeO}]$. ^1H NMR (CDCl_3): δ 7.533, 7.385, 4.771 (s, 2H), 3.381 (m, 2H), 3.127 (s, 6H), 1.748 (s, 2H), 1.234 (t, 20H), 0.832 (t, 3H). ^{13}C NMR (CDCl_3): δ 133.12, 130.65, 129.18, 127.37, 67.82, 63.94, 53.43, 49.45, 31.85, 29.35, 26.3, 22.83, 22.62, 14.06. A new peak for $[\text{ADBA}][\text{Me}]$ was observed at 53.43 (^{13}C NMR spectra for methoxide carbon). New ^{13}C NMR peaks for $[\text{ADBA}][\text{tBuO}]$ were observed at 73.74 and 84.10 (^{13}C NMR spectra for t-butoxide anion), respectively.

CATALYST CHARACTERIZATION

The EFTEM images depict the catalysts containing palladium nanoparticles in an ionic liquid layer immobilized on ACC (Figures 2, 3). In a fresh catalyst containing the ionic liquid

$[\text{ADBA}][\text{MeO}]$, the palladium particle size is below 10 nm whereas the particle size of spent catalyst is over 20 nm (Figure 2). Similar agglomeration of palladium particles has been observed with various SILCA catalysts (Virtanen et al., 2007; Salminen et al., 2012). Consequently, this might be one important reason to the deactivation of the catalysts. However, ionic liquids suppress agglomeration. This can be observed when comparing ionic liquid modified catalysts to unmodified catalysts. Palladium particle size can be over 100 nm with the spent Pd/ACC catalyst (Virtanen et al., 2007).

The results from nitrogen physisorption measurements are illustrated in Table 1. The micropore volume of pure ACC was in the range of $0.6 \text{ cm}^3/\text{g}$ whereas the specific surface area of pure ACC was $1680 \text{ m}^2/\text{g}$. Smaller surface areas and pore volumes of the spent catalysts with respect to the fresh catalysts indicated that some products and by-products are accumulating into the ionic liquid layer. This is one of the primary reasons for catalyst deactivation. Similar accumulation effects have been observed in the hydroformylation of propene over supported ionic liquid phase catalysts (Riisager et al., 2005). Moreover, accumulation of citral hydrogenation products to catalyst media was observed when the spent catalysts were extracted using methanol.

CITRAL HYDROGENATION

The hydrogenation of citral was studied using two alkaline ionic liquids immobilized on the catalyst support. These alkaline ionic liquids were synthesized from $[\text{ADBA}][\text{Cl}]$ and sodium methoxide or potassium tert-butoxide. The catalysts containing alkaline ionic liquids were compared to a catalyst containing $[\text{ADBA}][\text{Cl}]$ and to a traditional heterogeneous catalyst, consisting of palladium nanoparticles on ACC. Alkaline ionic liquids used in SILCAs were $[\text{ADBA}][\text{MeO}]$ and $[\text{ADBA}][\text{tBuO}]$. The plausible citral hydrogenation reaction scheme is presented in Figure 4.

The most active catalyst was Pd in $[\text{ADBA}][\text{tBuO}]$ on ACC. This supported ionic liquid catalyst was over three times as active as the traditional heterogeneous catalyst (Figure 5). SILCA

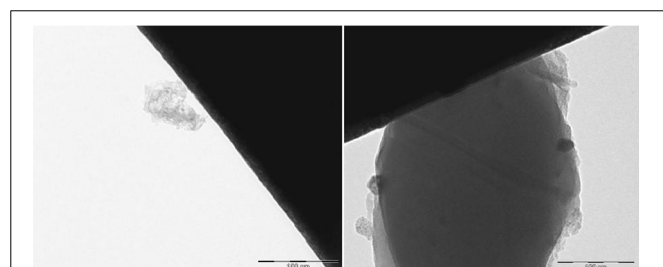


FIGURE 2 | EFTEM pictures of the catalyst containing palladium in a (ADBA)(MeO) on ACC as fresh (left) and after being exposed to citral hydrogenation reaction.

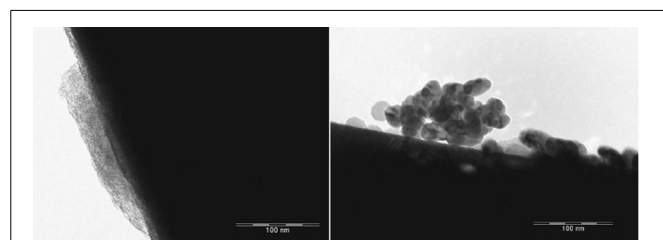


FIGURE 3 | EFTEM pictures of the catalyst containing palladium in (ADBA)(tBuO) on ACC as fresh (left) and after being exposed to citral hydrogenation procedure.

Table 1 | Results from the nitrogen physisorption measurements.

Catalyst		Surface area (m^2/g) ^a	Micropore volume (cm^3/g) ^b
Pd/[ADBA][MeO]/ACC	Fresh	717	0.26
	Spent	106	0.04
Pd/[ADBA][tBuO]/ACC	Fresh	956	0.34
	Spent	82	0.03

^a Calculated by Dubinin method.

^b Calculated by Dollimore/Heal method.

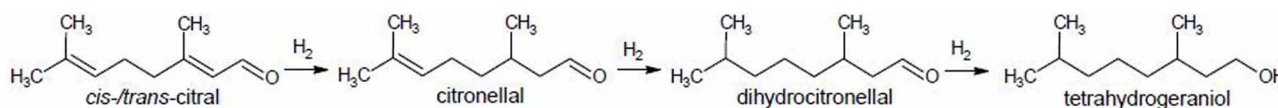


FIGURE 4 | Citral hydrogenation reaction sequence using alkaline ionic liquids in supported ionic liquid catalysts.

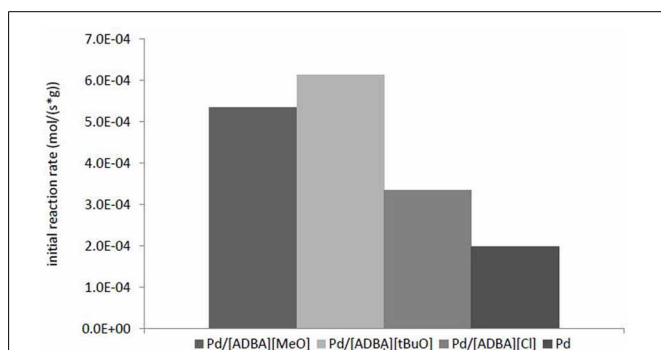


FIGURE 5 | Comparison of the initial reaction rates with various supported ionic liquid catalysts. The initial reaction rates were calculated at 5 min from commencing the reaction. All reactions presented here were carried out at $T = 100^{\circ}\text{C}$ and $p(\text{H}_2) = 10$ bar. The catalysts were Pd in [ADBA][Cl], [ADBA][MeO], and [ADBA][tBuO] on ACC. Pd on ACC is the traditional heterogeneous reference catalyst.

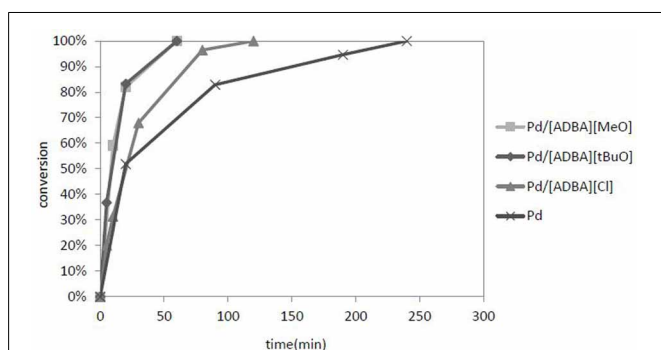


FIGURE 6 | A graph of citral conversion to products as a function of time. The catalysts were Pd on ACC and Pd in different ionic liquids on ACC. The ionic liquids were [ADBA][Cl], [ADBA][MeO], and [ADBA][tBuO], respectively. The reaction conditions were $T = 100^{\circ}\text{C}$, $p(\text{H}_2) = 10$ bar.

containing alkaline ionic liquids were the most active catalysts. Moreover, the conversion of citral was faster when using catalysts containing alkaline ionic liquids. The total conversion of citral was reached four times faster with SILCAs containing alkaline ionic liquids than when traditional heterogeneous catalyst (Pd on ACC) was applied (Figure 6).

As observed, SILCA consisting of alkaline ionic liquids demonstrated enhanced selectivities toward citronellal. When the catalyst was containing the ionic liquid [ADBA][MeO], the main product obtained was citronellal. When using alkaline ionic liquids in SILCAs, the only hydrogenation products were citronellal, dihydrocitronellal, and tetrahydrogeraniol. Citronellal and dihydrocitronellal were the main products (Figure 7). Other products were compounds from cracking and dehydration of citral and its hydrogenation products. In the first phase, the hydrogenation reaction proceeds almost totally toward citronellal by hydrogenating the conjugated carbon-carbon double bond. From citronellal, the reaction proceeds to dihydrocitronellal and further to tetrahydrogeraniol. Further, in the case of the catalyst containing the alkaline ionic liquid [ADBA][MeO], the molar yield of citronellal increased from 50 to 70% when the hydrogen pressure was lowered from 10 to 5 bar. The increase in citronellal molar yield is predictable, since lowering the hydrogen pressure increases the yield of less hydrogenated, intermediate products (e.g., citronellal). The time required for total conversion increased when pressure was changed from 10 to 5 bar.

Claus et al. demonstrated that the Pd/C catalyst coated with the alkaline dicyanamide based ionic liquid ($[\text{C}_4\text{C}_1\text{IM}][\text{N}(\text{CN})_2]$) is highly selective toward citronellal (Arras et al., 2008). In our experiments (100°C and 20 bar) the activity of the catalyst Pd in 1-butyl-3-methylimidazoliumdicyanamide ($[\text{C}_4\text{C}_1\text{IM}][\text{N}(\text{CN})_2]$) on ACC was very poor, even though the selectivity toward citronellal was high (9% conversion and 90% selectivity in 24 h).

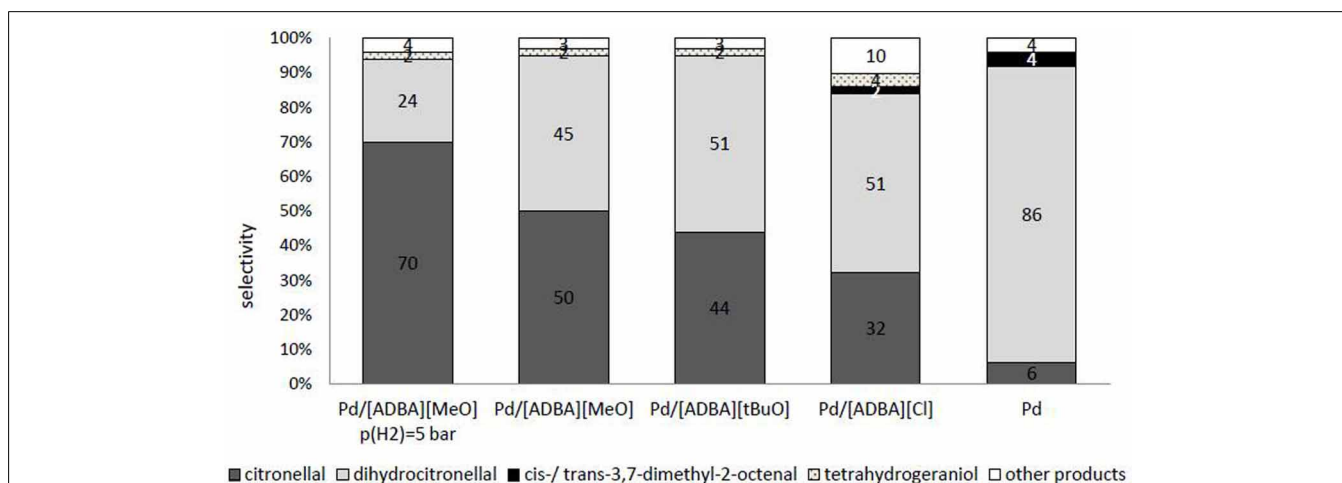


FIGURE 7 | Comparison of the selectivities of the products from citral hydrogenation over supported ionic liquid catalysts containing an alkaline ionic liquid after complete conversion. Traditional heterogeneous catalyst (palladium nanoparticles on ACC) and catalyst

containing [ADBA][Cl] were chosen as reference catalysts. The reaction conditions were $T = 100^{\circ}\text{C}$, $p(\text{H}_2) = 10$ bar. Other products are compounds from cracking and dehydration of citral and its hydrogenation products.

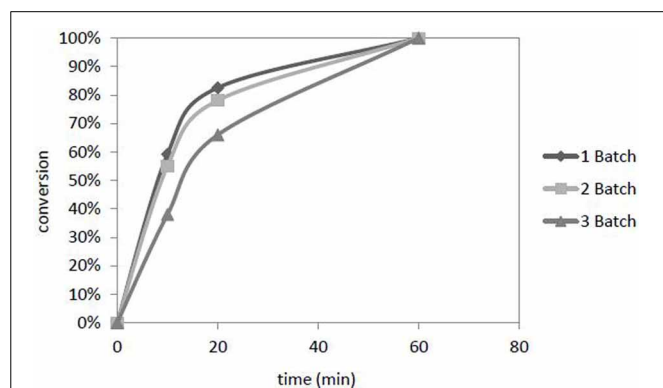


FIGURE 8 | Deactivation of the catalyst Pd in [ADBA][MeO] on ACC during three consecutive experiments. Citral conversion to products is presented as a function of time. The reaction conditions were $T = 100^{\circ}\text{C}$, $p(\text{H}_2) = 10$ bar.

The catalyst Pd/[ADBA][MeO]/ACC was reused three times in consecutive batches in the transformation of citral (Figure 8). The temperature and pressure were 100°C and 10 bars in every batch, respectively. The plausible reasons for catalyst deactivation are the accumulation of citral hydrogenation products into the ionic liquid layer and agglomeration of palladium particles on the catalyst surface as shown in catalyst characterization section.

With SILCA containing alkaline ionic liquid ([ADBA][MEO]), 70% molar yield of citronellal was obtained whereas alkaline-modified (e.g., KOH) SILCAs, studied in our earlier work, resulted in 74% citronellal molar yields (Salminen et al., 2012). It was observed that SILCAs containing alkaline ionic liquids could be used as a more feasible and catalytically stable alternative to alkaline modified SILCAs. The deactivation of alkaline modified SILCAs is much higher than in SILCAs containing alkaline ionic liquids.

CONCLUDING REMARKS

The increase in activity and citral conversion were noticed in SILCAs containing alkaline ionic liquids. Consequently, we propose that these strong alkaline ionic liquids take the role of a basic promoter, which was the case when e.g., potassium hydroxide was applied as a promoter leading to higher reaction rates and selectivities toward citronellal. The most active catalyst, Pd in [ADBA][tBuO] on ACC, was over three times as active as the traditional heterogeneous catalyst. In the case of the SILCA containing the ionic liquid [ADBA][MeO] and upon a hydrogen pressure of 5 bars, the main product was citronellal (yield 70%). Highly selective reaction route toward citronellal was accomplished when applying alkaline ionic liquids in SILCAs.

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Natural additives and agricultural wastes in biopolymer formulations for food packaging

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The main directions in food packaging research are targeted toward improvements in food quality and food safety. For this purpose, food packaging providing longer product shelf-life, as well as the monitoring of safety and quality based upon international standards, is desirable. New active packaging strategies represent a key area of development in new multifunctional materials where the use of natural additives and/or agricultural wastes is getting increasing interest. The development of new materials, and particularly innovative biopolymer formulations, can help to address these requirements and also with other packaging functions such as: food protection and preservation, marketing and smart communication to consumers. The use of biocomposites for active food packaging is one of the most studied approaches in the last years on materials in contact with food. Applications of these innovative biocomposites could help to provide new food packaging materials with improved mechanical, barrier, antioxidant, and antimicrobial properties. From the food industry standpoint, concerns such as the safety and risk associated with these new additives, migration properties and possible human ingestion and regulations need to be considered. The latest innovations in the use of these innovative formulations to obtain biocomposites are reported in this review. Legislative issues related to the use of natural additives and agricultural wastes in food packaging systems are also discussed.

Keywords: additives, active packaging, nano-biocomposites, legislation as topic, food wastes

INTRODUCTION

The food packaging industry is showing increasing interest in the development of new materials and processes to ensure that the food contained is safe and healthy and to improve shelf-life, which is an essential issue in the modern distribution and commercialization strategies. In addition, the use of commodities has greatly increased in the last decades in food packaging applications. Among the wide variety of materials currently used in food packaging, polymers have taken a major share because of their versatility and advantageous performance/cost ratio. However, most polymers used for food packaging are not biodegradable or compostable and therefore represent an increasingly serious end-of-life disposal problem worldwide. Although food package stability during the shelf-life of the product is an advantage, it turns into a disadvantage when the packages enter the post-use phase. In consequence, the use of polymeric materials obtained from non-renewable resources (which are usually non-biodegradable) in food packaging applications presents important environmental impact and waste generation issues. Packaging waste accounted for 71.6 million tons or 29.5% of the total municipal solid waste (MSW) in 2009 in the USA (<http://www.epa.gov/osw/nonhaz/municipal/pubs/msw2009-fs.pdf>) Accessed June 2011) and 56.3 million tons or 25% of the Municipal Solid Waste (MSW) in 2006 in Europe (<http://www.waste-management-world.com/index/display/article-display/304406/articles/waste-management-world/volume-8/issue-4/features/msw-management-in-europe.html>) Accessed June 2011).

Currently, landfilling is the dominant method of packaging waste disposal, followed by recycling, incineration, and composting. Even though recovery methods such as reuse, recycling and/or composting are encouraged as a way of reducing packaging waste disposal, there is still much work to do to substantially reduce the quantity of plastic present in MSW (Jamshidian et al., 2010). Nevertheless, commercial biopolymers show some limitations in terms of performance (thermal resistance, poor barrier, and brittleness) as well as relatively high prices, which are limiting their current applications, requiring modification by addition of other components to improve their performance in food packaging applications.

One of the most studied issues when talking about a package, not just looking for a simple container but also to improve the foodstuff characteristics, are listed below:

- (1) Allowing a slow but controlled respiration (reduced O₂ absorption) of foodstuff through the packaging material;
- (2) Allowing a selective barrier to gases (in particular CO₂) and water vapor;
- (3) Creating a modified atmosphere with respect to the internal gas composition, regulating the ripening process, and leading to food shelf-life extension;
- (4) Lowering the lipids migration, avoiding the packaging modification;
- (5) Maintaining the polymers structural integrity to improve mechanical handling and processing;

- (6) Serving as a vehicle to incorporate food additives (flavor agents, colorants, antioxidants, antimicrobial agents);
- (7) Preventing (or reducing) microbial spoilage during extended storage and distribution (Tharanatha, 2003).

Food industry, and particularly all these companies producing materials for packaging, is subject to pressure from different stakeholders in the production and distribution chain. Producers, retailers and customers have different priorities, and packaging is not always perceived as adding value to the product. But food industry should invest time and energy in defending of packaging with reduced waste across the whole supply chain (Barlow and Morgan, 2013). This is the main reason to think on the use of agricultural and food processing wastes in the packaging industry, while many research institutions have focused their strategy consisting of two issues:

- Reduction in environmental pollution by the packaging industry
- Recovery of biopolymers to use them in the preparation of biodegradable materials (Prochoń and Przepiórkowska, 2013).

It is well known that bio-based and biodegradable polymers can be classified in four categories depending on the synthetic route followed to get them (Vieira et al., 2011).

1. Polymers obtained from biomass, in particular from agro-resources, such as polysaccharides, e.g., starches (wheat, potatoes, maize) ligno-cellulosic products (wood, straws) and others (pectins, chitosan/chitin, gums) protein and lipids, e.g., animals (casein, whey, collagen/gelatin), and plants (zein, soya and gluten).
2. Polymers obtained by microbial production, e.g., poly(hydroxyalkanoates) (PHAs) such as poly(hydroxybutyrate) (PHB) and poly(hydroxybutyrate co-hydroxyvalerate) (PHBV);
3. Polymers chemically synthesized using monomers obtained from agro-resources, e.g., poly(lactic acid) (PLA);
4. Polymers whose monomers are obtained by chemical synthesis from fossil resources, e.g., poly(ϵ -caprolactone) (PCL), poly(esteramides) (PEA), aliphatic co-polyesters (e.g., PBSA) and aromatic co-polyesters (e.g., PBAT).

The most widely studied thermoplastic biopolymers are starch, PLA and PHAs. Among these, starch and PLA biopolymers are the most interesting for food packaging since they have become commercially available, have an interesting balance of properties, and are produced on an industrial scale. For instance, PLA shows excellent transparency and relatively good water resistance (Park et al., 2002; Chen et al., 2003; Tsuji and Yamada, 2003). Its high stiffness is usually reduced by the addition of plasticizers (Martin and Averous, 2001; Ljungberg and Wesslén, 2002, 2003), but these additives also lead to a decrease in oxygen barrier and thermal resistance (Martino et al., 2009; Courgneau et al., 2011). It is therefore of great industrial interest to enhance the barrier properties of PLA while maintaining its inherently good properties such as transparency and biodegradability. This is one of the

main challenges at present in the development of biocomposites for food packaging applications.

There are other biomaterials with a high potential in food packaging and which can be directly extracted from biomass, such as gluten, zein, prolamine obtained from corn and chitosan, which is typically obtained from crustacean chitin. These materials generally have excellent oxygen barrier under dry conditions. However, their main drawbacks are their inherent high rigidity, difficulties in processing using conventional equipment and strong water sensitivity arising from their hydrophilic character, which leads to plasticization affecting mechanical and barrier properties (Anderson and Lamsa, 2011; Rogovina et al., 2011). Nevertheless, chitosan and zein biopolymers exhibit two very interesting characteristics. Chitosan has antimicrobial properties (Aider, 2010; Campos et al., 2011) and the unusual water resistance of zein makes this biopolymer potentially useful for multilayer food packaging applications (Wang and Padua, 2004).

All biopolymers with commercial interest can show excellent gas barrier properties in their optimum formulations, although their barrier performance is dramatically reduced in the presence of moisture and/or other plasticizers, necessary to get materials adequate for processing. Other biopolymers like PHAs, show very high water barrier properties. So, in principle, the use of multilayer systems where an inner layer of plasticized chitosan could be sandwiched between PHA layers could be an interesting possibility. However, these materials normally suffer from relatively high production costs, meaning that competition with conventional thermoplastics is still problematic. Therefore, the addition of natural additives and/or agricultural waste by-products discarded from food processing operations is an innovative trend in polymer science with clear practical interest.

In this review we summarize these new approaches and the main recent developments in this area are presented.

USE OF NATURAL EXTRACTS IN BIODEGRADABLE POLYMERS

New developments in food packaging materials have been recently implemented by the increase in consumers demand for minimally processed foods and stricter requirements regarding consumer health and safety. In fact, new technologies are being investigated in this research line, such as modified atmosphere packaging and active packaging technologies (Suppakul et al., 2003).

Different natural compounds have been proposed for incorporation to the polymer matrices to improve the packaging's functionalities as well as the food quality and safety. Their action is essential in reducing or even eliminating some of the main food spoilage causes, such as rancidity, color loss/change, nutrient losses, dehydration, microbial proliferation, senescence, gas build-up, and off-odors (López-Gómez et al., 2009).

The demand for the use of natural additives in polymer formulations has produced in recent years a clear increase in the number of studies based on natural extracts which come from plants, essential oils or agricultural waste products (Coma, 2008; Gutiérrez et al., 2009a; Kuorwel et al., 2011). A large number of studies have described the broad antimicrobial spectrum against different pathogenic and spoilage microorganisms,

including Gram-negative and Gram-positive bacteria and molds. For instance, extracts of blueberry obtained from four cultivars have shown an antimicrobial effect on the growth of *Listeria monocytogenes* and *Salmonella Enteritidis* (Shen et al., 2014). Grape seed extracts can be used as additives for its well documented anti-inflammatory and antimicrobial properties against major food borne pathogens like *L. monocytogenes*, *Salmonella Typhimurium*, *Escherichia coli* (*E. coli*), and *Campylobacter Jejuni* in preventing pathogen contamination. A green tea extract can inhibit the growth of various strains of *Staphylococcus* and some Gram negative bacteria, such as *E. coli* or *Salmonella* (Perumalla and Hettiarachchy, 2011). A recent study demonstrated the promising antimicrobial effects on food of three extracts from raspberry fruits and pomace, including against *E. coli*, *Salmonella sp.*, *L. monocytogenes*, *Enterococcus Faecium* (Caillet et al., 2012). These additives are considered to be safe and have the “Generally Recognized As Safe” (GRAS) status as designated by the American Food and Drug Administration (Kuorwel et al., 2011). A summary of the most relevant additives extracted from natural sources is shown in **Figure 1**.

Due to these beneficial properties, the use of natural extracts or their original compounds (including low molecular weight phenolic acids, tannins, proanthocyanidins, flavonoids, such as anthocyanins or flavonols) for food packaging applications is growing in the last years (Smith-Palmer et al., 2001; Bañón et al., 2007; Carpenter et al., 2007; Negi, 2012). But, sometimes these chemicals are too volatile to be directly incorporated to foodstuff. For this reason a viable alternative is to incorporate these compounds into packaging materials as natural active additives with the possibility to be released to food (Burt, 2004; Del Nobile et al., 2009; Gutiérrez et al., 2009a). In this sense, the antimicrobial active substances are incorporated into the polymer matrix and then they can be gradually released from the packaging material onto the food surface during the total shelf-life of the packaged foodstuff increasing their effect with the aim to control the minimum inhibitory concentration needed for bacterial growth inhibition (Gutiérrez et al., 2009a).

Different types of biopolymers can be used in the development of antimicrobial active films. For instance, Iturriaga et al. used two different biopolymer matrices (gelatin and methyl cellulose) to develop antimicrobial active edible films with citrus extract. These formulations showed convenient properties (lack of odor, water solubility) and interesting antibacterial activity shown against three bacteria by using the agar diffusion method, measuring the inhibition halo against the tested microorganisms. These results showed that the inhibitory effectiveness of the films against the tested strains was maintained regardless of the biopolymer matrix for at least one month (Iturriaga et al., 2012).

Other edible films as soy protein isolate formulations containing grape seed extracts were developed by Sivarrooban et al. who demonstrated that these biocomposites were able to reduce the populations of bacteria related with food (*E. coli* O157:H7 and *Salmonella Typhimurium* were reduced by 1.8 and 0.6 log CFU/mL, respectively). The incorporation of these extracts significantly increased the films thickness (from 33.0 to 77.9 μm), puncture strength (from 2.5 to 5.3 N), and tensile strength (8.8–10.7 MPa) in comparison to the pure soy protein isolate film

(Sivarrooban et al., 2008). Tensile properties could be improved by the addition of the natural extracts due to their intercalation in the polymer matrix chains resulting in higher mechanical resistance. Another example was the addition of grapefruit seed extracts to obtain active films based on barley bran protein and gelatin to pack salmon fish. After 15 storage days results showed a significant decrease in the populations of *E. coli* O157:H7 and *L. Monocytogenes* compared to the control neat materials (Song et al., 2012). The combination of mint extracts or pomegranate peel extracts with chitosan and polyvinyl alcohol resulted in an improvement of their tensile strength without significantly affecting their barrier properties and effectiveness against Gram-positive food bacteria (Kanatt et al., 2012).

Synthetic biodegradable polyesters, such as PCL, were also used as polymer matrices to produce active food packaging films with the combination of natural extracts which could come from plants or agricultural waste. For instance, Takala et al. based their study in the development of PCL/Alginate films containing three natural extracts from rosemary and Asian and Italian essential oils. The potential in controlling/inhibiting the growth of food borne pathogens in fresh-cut vegetable was studied. Results showed that these composites might be affected by relative humidity in packaging, with the result of altering the mechanism of release of volatile compounds and consequently their antimicrobial activity and films properties (Takala et al., 2013).

Other natural extracts were tested by the need of increasing the value of some sub-products or by-products coming from agricultural waste or food processing residues. For instance, lemon extracts were used by Del Nobile et al. to develop active materials for packaging based on PLA, PCL, and LDPE. In this case the processing temperature of PCL played an important role, since it was necessary to reduce it drastically to avoid the thermal degradation of the polymer matrix and to reduce the risk of the inactivation of the active compounds or volatilization of the lemon extracts (Del Nobile et al., 2009). Propolis extracts were tested in combination with PLA by Mascheroni et al. with the aim to develop an anti-microbial/antioxidant release system for food packaging (Mascheroni et al., 2010). Olive leaves are other important source of active compounds, and the extract showed high antimicrobial efficiency, caused by their high concentration in polyphenols. For this reason, Erdohan et al. studied the incorporation of olive leaf extracts into a PLA matrix. Antimicrobial tests showed that the increase in the amount of the extract could cause a significant increase in inhibitory zones. Moreover, the water vapor permeability, the water solubility, and the degradation rates were modified with the increase of the extracts concentration (Özge Erdohan et al., 2013).

An alternative to composite materials in formulations for food packaging is the use of multilayer systems based on biodegradable layers in combination with natural extracts. Thus, Takala et al. developed trilayer composite films based on methylcellulose (MC) and PCL. The incorporation of natural extracts, such as rosmarinic acid, showed their potential application to control food pathogens, as well as a clear decrease of barrier properties and no significant modifications on tensile strength (Takala et al., 2013).

The addition of natural additives to polymer matrices for food packaging allows their sustained release (migration) to foodstuff

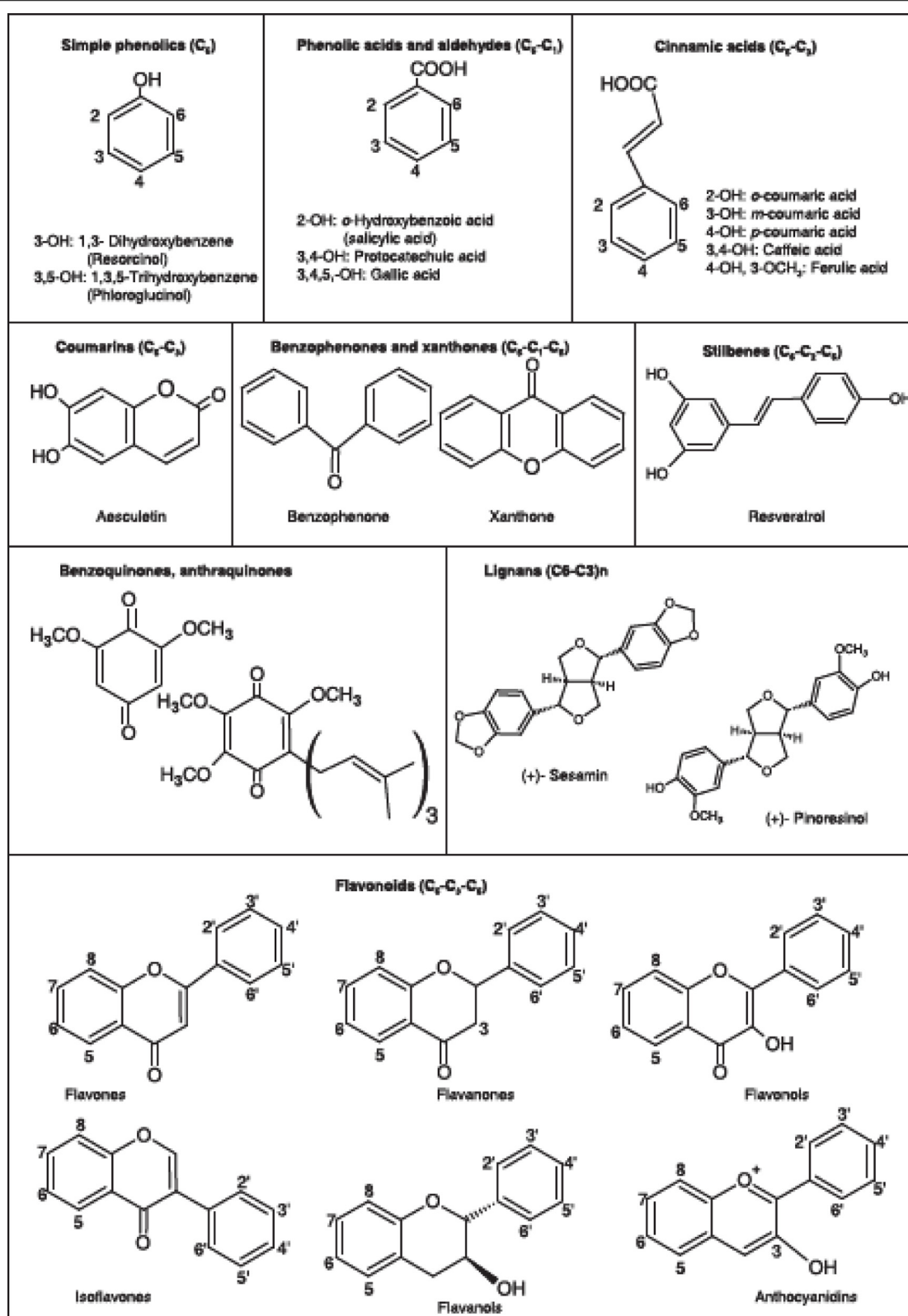


FIGURE 1 | Phenol derivatives (C_6) commonly found in industrial wastewaters or fractions isolated from vegetal sources. Representative examples are indicated under the general chemical structures (Soto et al., 2011) (with permission).

during long periods of time (including storage and distribution operations), extending the shelf-life of the final product by the decrease in food autooxidation or spoilage, affecting both sensory and nutritional qualities of food (Manzanarez-López et al., 2011). Migration is the result of diffusion, dissolution, and equilibrium processes involving the mass transfer of low molecular mass compounds initially present in the package into a food sample or food simulant; and it is often described by Fick's second law (Manzanarez-López et al., 2011). The determination of migration of additives in food samples is, in most cases, time consuming and expensive. Thus, migration studies are usually performed using food simulants and conditions specified in European food packaging regulations (Kuorwel et al., 2013).

REVALORIZATION OF AGRICULTURAL RESIDUES AS REINFORCEMENT IN BIOPOLYMERS

The fabrication of green composites formed by biopolymers and natural fibers has attracted major interest in polymers and composites research. The public concern about the environment, climate change and global warming while limited fossil fuel resources are available, has been important driver for governments, companies and scientists to find alternatives to crude oil in plastics production. Bio-composites obtained from biopolymers and reinforced with natural fibers may offer important contributions by reducing the dependence on fossil fuels and the related environmental impacts. In particular, the use and valorization of agricultural waste is currently a trending topic in research and a raising number of results in this area are being reported.

Natural fibers are materials extracted from substances in Nature that can be classified into three main categories: vegetable (plant fibers), animal and mineral fibers (**Figure 2**). Vegetable or plant fibers are classified into three main subdivisions: leaf fibers (e.g., oil palm, banana, sisal, pineapple, abaca leaf . . .); bast fibers (e.g., kenaf, jute, and flax); seed fibers (e.g., cotton, rice husk, kapok, and coir). Fibers can be considered as naturally occurring composites constituted mainly of holocellulose (cellulose, hemicellulose) and lignin, with minor contents of sugars, starch, proteins, extractives, and ash (Hamza et al., 2013). The unidirectional cellulose microfibrils constitute the reinforcing elements in the matrix blend of hemicellulose and lignin (Faruk et al., 2012). Several advantages of incorporating natural fibers into biopolymer matrices are interesting to note, since due to their low density and cost, availability, recyclability, environmental friendliness, total degradation in soil without emission of toxic compounds in composting condition, and good mechanical properties, have permitted scientists worldwide to show interest in exploiting the full potentials of plant fibers.

In recent years, researchers have been involved in valorizing agricultural residues and lignocellulosic fibers as polymer reinforcements in substitution of those obtained from glass and carbon. For example, the outer covering of the hull of the soybean seed is a by-product generated during the soybean oil processing and this residue was demonstrated as a good reinforcement to obtain engineer green composites in poly(3-hydroxybutyrate-co-valerate) and polylactide blends (60:40 wt%) (Nanda et al., 2013). This study showed that the addition of 30% of soybean hull induced an important increase in tensile modulus of

these composites that may be attributed to the high stiffness of fibers, indicating the efficient transfer of stress from the matrix to the fiber phase; hence an efficient stress transformation is expected in these composites. The potential use of incorporating lignocellulosic agricultural residues, such as soy stalk, corn stalk, wheat straw, and perennial grasses, like switchgrass and miscanthus, as reinforcement into a biodegradable polymer matrices comprising a poly(3-hydroxybutyrate-co-3-hydroxyvalerate) and poly(butylene adipate-coterephthalate) blend has been recently investigated (Nagarajan et al., 2013). The incorporation of miscanthus, with high cellulose amounts which allowed better interactions with the matrix, resulted in increased tensile strengths of composites. The flexural strength of these composites with lignocellulosic fibers obtained from agricultural wastes improved significantly, with the exception of the switchgrass-based composite. Finally, the moduli of these green composites increased drastically with no significant differences between fiber types. However, the elongation at break of bio-composites was found to be lower than 5% regardless of the bio-fiber type and due to the restriction of the molecular mobility of the polymer chains in the composite. In addition, the impact strength was reduced by two mechanisms: (1) the drastic reduction in elongation at break, reducing the area under the stress-strain curve, and (2) the stress concentration at areas of poor adhesion around fiber ends and fiber-fiber contacts.

A preview review (Wahit et al., 2012) highlighted developments in the production of PLA and PCL eco-friendly composites with enhanced mechanical properties and biodegradation with the incorporation of natural fibers such as hemp, wood, ramie, kenaf, rice straw, abaca, jute, bamboo, rice husk, oil palm, and flax. In this sense, a comparative study of the mechanical properties of PLA/kenaf and PLA/rice husk composites showed the remarkable increase in flexural modulus. For the PCL/flax fiber composite, tensile strength, and flexural strength increased by 54 and 44%, respectively. PCL/rice husk composite decreased in melting temperature and crystallinity at high fiber contents. Moreover, the high-level degradation for all PCL/rice husk composites after 57 days of incubation was achieved since the addition of rice husk facilitated the access of water to the PCL matrix as well as the controlled absorption and stabilization of PCL depolymerase, resulting in a clear increase of the PCL hydrolytic degradation. The morphology, crystallization behavior, thermal, mechanical and barrier properties and biodegradation in soil of fully biodegradable bio-composites with 5 and 15 wt% of cotton, cellulose obtained from cotton and hydrolyzed cellulose into a PCL matrix was also investigated (Gutiérrez et al., 2009b). It was concluded that the incorporation of lignocellulosic fillers drove to a decrease on the tensile strength and elongation at break, as well as an increase on the elastic modulus. The cellulose obtained from cotton residues, due to their lower hydrophilicity, showed the highest compatibility with PCL, while strongly hydrophobic reinforcement fillers resulted in increased mechanical properties (Faruk et al., 2012). The best performance was obtained with the addition of 15 wt% of cellulose obtained from cotton residues, which showed the highest toughness and did not change the gas barrier properties of the neat matrix. The biodegradation processes were accelerated by the

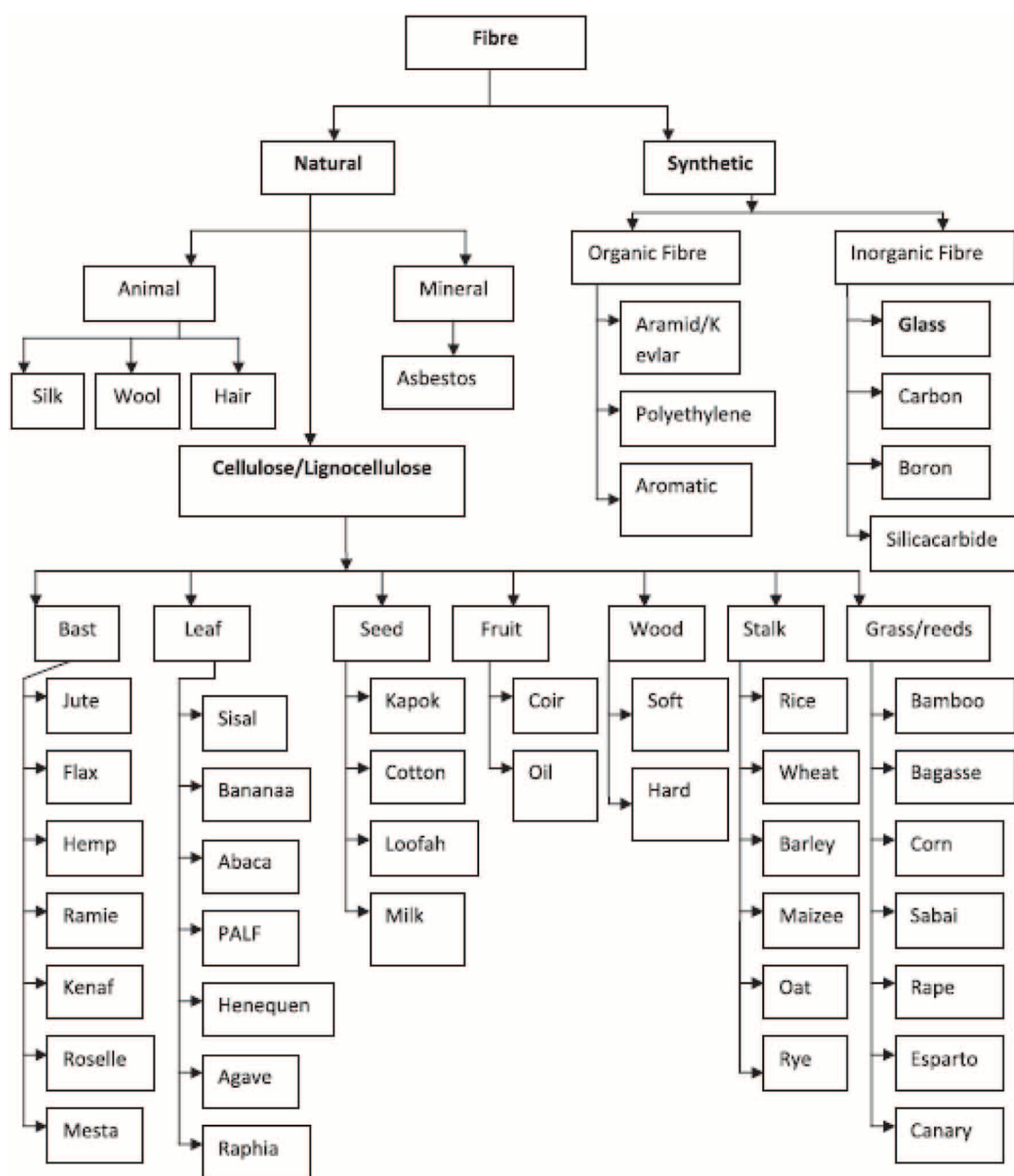


FIGURE 2 | Fibers classification (Majeed et al., 2013) (with permission).

presence of these fillers but the effect was not severe. Therefore, these composites could be useful for packaging applications. Similar behavior was reported in valorising different agricultural residues, such as walnut shells (Pirayesh et al., 2012), almond shells (Pirayesh et al., 2013), orange tree pruning fibers (Reixach et al., 2013), bamboo (Abdul Khalil et al., 2012), rice-husk fiber, bagasse fiber and waste fish in polypropylene (Nourbakhsh et al., 2014), jute, flax and wood, among others, as reinforcement in polymeric composite materials for different industrial applications.

Composites reinforced with agricultural residues have been object of raising interests in the development of active packaging systems. For example, ethylene-scavenging packaging materials have been subject to recent studies to be applied in the fresh fruit and vegetables industry. Commercial ethylene scavengers are normally based on potassium permanganate (KMnO_4) but a clear limitation on their application with regard to food is that KMnO_4 is not allowed to come into contact with foodstuff because of its toxicity and color. In order to overcome this problem, composites with rice straw incorporating activated carbon were

proposed and their effect on the physical and mechanical properties in the resulting composites was reported (Sothornvit and Sampoompuang, 2012). Results showed that the incorporation of 30% activated carbon to rice straw paper produced the maximum level of ethylene scavenging (77%) in environmentally-friendly way owing to its reusability and protecting against mechanical damage.

LEGISLATIVE ISSUES

Due to interactions with food and/or the environment, active packaging represents a new challenge to progress in advanced materials for research. The intentional migration of active elements through the packaging materials and to food would fall under the Framework Regulation 1935/2004 for active packaging materials (European Commission, 2004). This Regulation already contained general provisions on the safety of active packaging and set the framework for the EFSA's safety evaluation process (Dainelli et al., 2008). But some articles, such as active and intelligent materials, fall within more specific regulations. In this sense, the Commission Regulation (EC) No 450/2009 (European Commission, 2009) was related to active and intelligent packaging and it pointed out that the active element needs to be identified and the active material has to be accompanied by information on the permitted uses. The maximum quantity of substances released by the active component should be specified (Llorens et al., 2012).

It should be highlighted that the amount of active compounds released from packaging materials could exceed the overall migration requirements indicated in the EU or national legislations. The transfer of these active substances to food should not be included in the calculation of the overall migration limit (OML). However, passive parts should be covered by the specific legislation applicable to those materials, such as the EU Regulation 10/2011. Only the active components should be subjected to safety assessment by the EFSA (European Food Safety Authority) before they are authorized for their use, and a list of substances or group/combination of substances to be used in active and intelligent materials should be drawn up following the risk assessment of these substances by EFSA.

Some Guidance to the Commission Regulation (EC) No 450/2009 was provided by the European Commission to deal with questions concerning the interpretation and implementation of certain aspects in the referred legislation (European Commission, 2011). This document contains some definitions and examples; clarification on packaging types and components that fall or not fall under the definition of active materials; legal aspects in related to the authorization of active substances or components; and questions and answers related to the risk assessment and authorization procedure by EFSA. Factors considered by the authority when making safety assessments include, for example, toxicological properties and the extent to which the original or their breakdown products could transfer into food.

EFSA guidelines are only applicable to substances in direct contact with food or the environment surrounding the food (headspace). These guidelines are also applied to materials which are separated from food by a functional barrier (i.e., a barrier consisting of one or more layers of food-contact materials which ensures that the finished material or article

Table 1 | List of some permitted food additives that could be used as active agents in packaging materials (Suppakul et al., 2003) (with permission).

Additive	Code assigned by legislative authority		
	Australia/ New Zealand ¹	Europe ²	USA ³
Acetic acid	260	E260	GRAS
Benzoic acid	210	E210	GRAS
Butylated hydroxyanisole (BHA)	320	E320	GRAS
Butylated hydroxytoluene (BHT)	321	E321	GRAS
Carvarcol			FA
Citral			GRAS
Citric acid	330	E330	GRAS
<i>p</i> -Cresol			FA
EDTA			FA
Estragloe (methyl chavicol)			GRAS
Ethanol		E1510	GRAS
Ethyl paraben		E214	GRAS
Eugenol			GRAS
Geraniol			GRAS
Glucose oxidase	1102		GRAS
Hexamethylenetetramine (HMT)		E239	
Konjac glucomannan		E425	GRAS
Lactic acid	270	E270	GRAS
Lauric acid			FA
Linalool			GRAS
Lysozyme	1105	E1105	GRAS
Mallic acid	296	E296	GRAS
Methyl paraben	218	E218	
Natamycin	235	E235	FA
Nisin	234	E234	GRAS
Phosphoric acid	338	E338	GRAS
Polyphosphate		E452	GRAS
Potassium sorbate	202	E202	GRAS
Propionic acid	280	E280	GRAS
Propyl paraben	216	E216	GRAS
Sodium benzoate	211	E211	GRAS
Sorbic acid	200	E200	GRAS
Succinic acid		E363	GRAS
Sulfur dioxide	220	E220	GRAS
Tartaric acid	334	E334	GRAS
Teritary butylhydroquinone (TBHQ)	319		FA
α -Terpineol			FA
Thymol			FA

Source: CFR (1988); Davidson and Branen (1993); Maga and Tu (1995); Luck and Jager (1997); Saltmarsh (2000); Taubert (2000).

¹ Assignment of a number signifies that additive is approved by the Australian and New Zealand Food Authority (ANZFA) and The Australian New Zealand Food Standards Council (ANZFSC) as being safe for food use.

² Assignment of an "E" number signifies that additive has been approved by the European Communities (EC), Scientific Committee on Food (SCF).

³ Classification in accordance with Food and Drug Administration (FDA) Title 21 of the code of Federal Regulations (21 CFR) wherein substances intended for use in the manufacture of foodstuffs for human consumption are classified into 3 categories: food activities (FA), prior-sanctioned food ingredients and substances generally recognized as safe (GRAS).

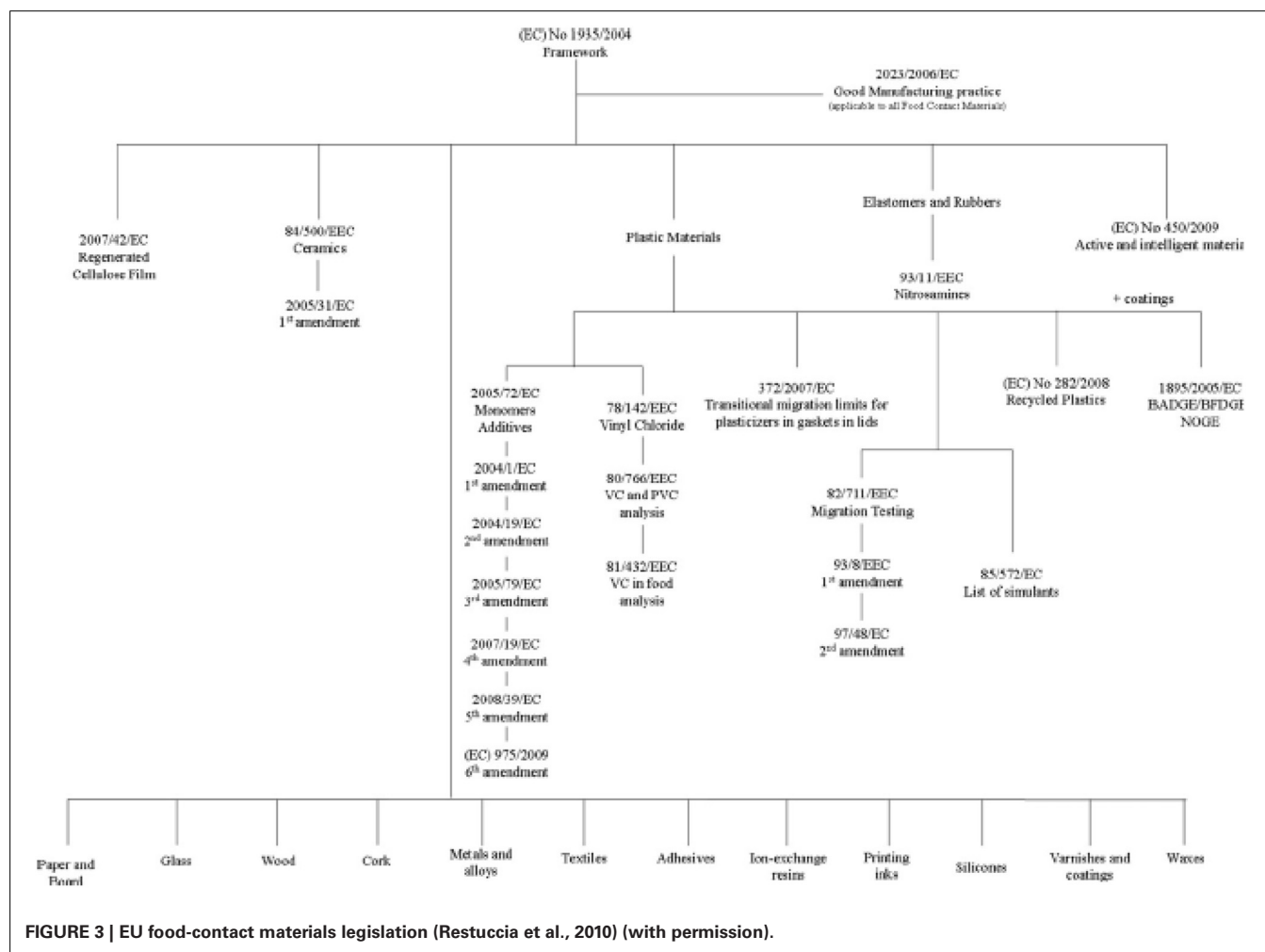


FIGURE 3 | EU food-contact materials legislation (Restuccia et al., 2010) (with permission).

complies with Article 3 of Regulation 1935/2004/EC and with Regulation 450/2009/EC). Substances behind such a barrier do not need a safety evaluation and are also outside the scope of these Regulations. Therefore, behind a functional barrier non-authorized substances may be used if their migration does not exceed 0.01 mg kg^{-1} food and they are not carcinogenic, mutagenic, or toxic to reproduction.

Considering regulatory requirements for new active packaging technologies in United States, materials used in food-contact applications are subject to pre-market regulatory clearance by the US Food and Drug Administration if they are deemed “food additives.” Some organic acids, bacteriocins and volatile compounds derived from plants have FDA approval as additives for certain foods (see Table 1) (Suppakul et al., 2003).

In summary, Regulations 1935/2004/EC and 450/2009/EC poses new basis for the general requirements and specific safety and marketing issues related to active packaging materials. In this sense, it should be considered that complexity of new developed systems introduce many variables into risk assessment; introducing new migration products and leading to different interactions between active agents and different packaging materials. The

development and validation of migration tests to reliably detect and measure new migration products could represent a serious challenge, as well as the risk assessment for nanomaterials (Restuccia et al., 2010). The main legislation associated with active materials is shown in Figure 3.

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The contributors of this book come from diverse backgrounds, making this book a truly international effort. This book will bring forth new frontiers with its revolutionizing research information and detailed analysis of the nascent developments around the world.

We would like to thank all the contributing authors for lending their expertise to make the book truly unique. They have played a crucial role in the development of this book. Without their invaluable contributions this book wouldn't have been possible. They have made vital efforts to compile up to date information on the varied aspects of this subject to make this book a valuable addition to the collection of many professionals and students.

This book was conceptualized with the vision of imparting up-to-date information and advanced data in this field. To ensure the same, a matchless editorial board was set up. Every individual on the board went through rigorous rounds of assessment to prove their worth. After which they invested a large part of their time researching and compiling the most relevant data for our readers.

The editorial board has been involved in producing this book since its inception. They have spent rigorous hours researching and exploring the diverse topics which have resulted in the successful publishing of this book. They have passed on their knowledge of decades through this book. To expedite this challenging task, the publisher supported the team at every step. A small team of assistant editors was also appointed to further simplify the editing procedure and attain best results for the readers.

Apart from the editorial board, the designing team has also invested a significant amount of their time in understanding the subject and creating the most relevant covers. They scrutinized every image to scout for the most suitable representation of the subject and create an appropriate cover for the book.

The publishing team has been an ardent support to the editorial, designing and production team. Their endless efforts to recruit the best for this project, has resulted in the accomplishment of this book. They are a veteran in the field of academics and their pool of knowledge is as vast as their experience in printing. Their expertise and guidance has proved useful at every step. Their uncompromising quality standards have made this book an exceptional effort. Their encouragement from time to time has been an inspiration for everyone.

The publisher and the editorial board hope that this book will prove to be a valuable piece of knowledge for researchers, students, practitioners and scholars across the globe.

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